THE PREVALENCE OF POLYMORPHISM OF EXON 3 EXPRESSION OF THE GROWTH HORMONE RECEPTOR GENE IN THE GENERAL POPULATION AND ITS INFLUENCE ON QUALITY OF LIFE, BODY COMPOSITION AND RESPONSE TO GROWTH HORMONE REPLACEMENT IN GROWTH HORMONE DEFICIENT ADULTS.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Medicine.

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DECLARATION

This thesis is the result of work performed whilst registered as a candidate for the degree of Doctor of Medicine at the University of Liverpool. I declare that no portion of the work in this thesis has been submitted elsewhere for another degree or qualification in any other university or higher institution of learning.

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Lastly but by no means the least, I give thanks to Almighty Allah (SWT) for His guidance and protection.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADH	Anti-diuretic hormone
AOGHD	Adult onset growth hormone deficiency
BC	Body composition
COGHD	Childhood onset growth hormone deficiency
DNA	Deoxyribonucleic acid
FSH	Follicle stimulating hormone
GH	Growth hormone
GHD	Growth hormone deficiency
GHR	Growth hormone receptor
GHR-*d3*	Homozygous isoform for exon 3 deletion of the growth hormone receptor gene
GHR-*d3/fl*	Heterozygous isoform for exon 3 deletion of the growth hormone receptor gene
GHR-*fl/fl*	Homozygous isoform for exon 3 expression of the growth hormone receptor gene
GHRH	Growth hormone releasing hormone
GST	Glucagon stimulation test
HPA	Hypothalomo-pituitary axis
IGF-1	Insulin-like growth factor 1
IGFBP3	Insulin-like growth factor binding protein 3
ITT	Insulin tolerance test
JAK2	Janus kinase 2

LBM	Lean body mass
LH	Luteinizing Hormone
MAPK	Mitogen-activated protein (MAP) kinases
mRNA,	Messenger Ribonucleic acid
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
PROP-1	Prophet of Pit1, paired-like homeodomain transcription factor
QoL	Quality of life
RhGH	Recombinant human growth hormone
SST	Somatostatin
STAT5	Signal transducer and activator of transcription

ABSTRACT

Title: The prevalence of polymorphism of exon 3 expression of the growth hormone receptor gene in the general population and its influence on the quality of life, body composition and response to growth hormone replacement in growth hormone deficient adults.

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Growth hormone (GH) deficiency developing in adult life results in impaired quality of life (QoL) and altered body composition (BC). As in children, it is now standard practice to treat adult growth hormone deficiency (GHD) with recombinant human GH (rhGH). Response to treatment with rhGH remains variable in patients with GHD. In health, the growth hormone receptor (GHR) is the principal regulator of growth hormone sensitivity and is an obvious candidate gene to influence the response to rhGH. A common polymorphism occurs in the GHR gene resulting in two isoforms of the GHR in humans generated either by expression or non-expression of exon 3 of the GHR gene. This results in 3 genotypes: full-length only with exon 3 expressed on both alleles i.e. homozygous GHR*fl*; short only with non-expression of exon 3 on both alleles i.e. homozygous *GHRd3* and a combination of the 2 isoforms i.e. heterozygous GHR-*d3/fl*. Population studies have documented that this polymorphism occurs in high frequency with the GHR-*d3/fl* genotype in 25-40% of subjects and the GHR-*d3/d3* genotype in 7-15%(1-3).

In children, polymorphism of exon 3 of the GHR has been shown to influence response to rhGH; children homozygous or heterozygous for exon 3 deletion, *d3/d3 or d3/fl* show better response to treatment, as demonstrated by a superior increase in height velocity.

There are studies in adult GHD populations on the effect of this common polymorphism on treatment based on IGF-1 response and other anthropometric measurements (4, 5). To date however, there are no studies investigating the effect of this polymorphism on QoL in

adults with GHD. In this project, we investigated adults with GHD who had been treated with rhGH for more than one year in order to determine the relationship between genomic deletion of exon 3 in the GHR gene and QoL, body composition (BC) and serum IGF-1 levels, to determine the effect on this polymorphism on QoL and to compare these variables to a healthy adult control population.

The results from the one hundred and seventy-three adult GHD patients studied in this project did not support our original hypothesis that patients with the *d3/fl* or *d3/d3* genotype are less likely to need rhGH therapy to improve quality of life than those with the fl/fl genotype.

AIMS OF THE THESIS

Primary:

1. To investigate the frequency of the three exon 3 genotypes in adult patients with GHD
2. To determine whether QoL and BC differ across GHR genotypes in adult patients with severe GHD on rhGH replacement therapy.

Secondary:

1. To establish the prevalence of the three exon 3 genotypes in a healthy adult control population from the Northwest of England.
2. To investigate relationships between exon 3 genotype, QoL and body composition in the control population

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

SECTION 1: THE PITUITARY GLAND - PHYSIOLOGY AND REGULATION OF THE PITUITARY HORMONES

Anatomy of the pituitary gland

The pituitary gland is a pea-sized organ located at the base of the brain beneath the hypothalamus. It is often considered the most important part of the endocrine system because it produces hormones that control many functions of other endocrine glands. Under-functioning of the pituitary gland, when there is underproduction of one or more of its hormones is called hypopituitarism.

The pituitary gland lies within a recess of the median part of the middle cranial fossa in the sphenoid bone (sella turcica) and is composed of two major components, the anterior lobe (adenohypophysis) and the posterior lobe (neurohypophysis) that can be readily distinguished radiologically by magnetic resonance imaging. The anterior lobe contains three subdivisions; the pars distalis, pars intermedia and pars tuberalis.

The pars distalis makes up the bulk of the anterior pituitary and is primarily responsible for the secretion of anterior pituitary hormones into the peripheral circulation. The pars intermedia lies between the pars distalis and the posterior pituitary and is vestigial in humans, while the pars tuberalis is well defined in most mammalian species and surrounds the infundibular stem. The floor of the sella, or lamina dura, abuts the sphenoid sinus, allowing direct surgical access to the pituitary by the transsphenoidal route. Other important boundaries to the pituitary gland are the cavernous sinus laterally, which contains the internal carotid artery surrounded with sympathetic fibres, and the cranial nerves III, IV, V (ophthalmic and maxillary branches), and VI. The optic chiasm is located superiorly, separated from the pituitary by the cerebrospinal fluid-filled suprasellar cistern and the dural roof of the pituitary, the diaphragma sella.

Embryologic Anatomy

The posterior lobe of the pituitary gland is smaller than the anterior lobe and embryologically derives from the neural primordia as an out-pouching from the floor of the third ventricle. Embryologically, the hypothalamus is derived from forebrain tissue and lies in the midline on the floor of the third ventricle. It is made up of multiple nuclei arranged in four regions (preoptic, supraoptic, tuberal & mammillary regions) and three zones: periventricular, medial and lateral zones(6). It is functionally and intimately linked to the pituitary gland through the rich portal circulation around the median eminence. The hypothalamic neurones synthesize hypophysiotropic releasing and inhibiting hormones directly into the portal system which influences the function of the anterior pituitary gland. In contrast, neurones from the supra-optic and tuberohypophyseal nuclei directly innervate the posterior pituitary gland (7). The pituitary gland lies within the pituitary fossa of the sphenoid bone above the sphenoid sinus. Embryologically and functionally the pituitary gland comprises two distinct parts. The epithelial portion, which forms the anterior pituitary, has its origin from the stomodeal ectoderm of the Rathke's pouch. The neural portion which forms the posterior lobe, pituitary stalk, and infundibulum arises along with the rest of the hypothalamus from the diencephalic forebrain (7).

The blood supply to the pituitary is in keeping with this dual origin. The anterior gland receives a majority of its blood supply from the hypothalamo-hypophyseal portal system with some remaining blood supply via the pituitary capsular vessels derived from the superior hypophyseal arteries. The neurohypophysis receives its blood supply from the inferior hypophyseal branches of the internal carotid artery. Venous drainage from the anterior pituitary is via the cavernous sinuses, principally into the petrosal sinuses and thence into the internal jugular veins.

Physiology of the Pituitary Gland

Microscopically, the anterior pituitary is composed of nests or cords of cuboidal cells organized near venous sinusoids lined with a fenestrated epithelium into which secretory products from the anterior pituitary are collected. Classically, five cell types and six secretory products of the anterior pituitary gland can be identified immunocytochemically including the somatotroph (GH), lactotroph (prolactin), corticotroph (adrenocorticotropic hormone, ACTH), thyrotroph (thyroid-stimulating hormone, TSH), and gonadotroph (luteinizing hormone, LH and follicle-stimulating hormone, FSH) cells. It is now recognized, however, that the anterior pituitary is vastly more complicated. In addition to morphological and physiological evidence for heterogeneity among the classical anterior pituitary cell types and the presence of clusters of a unique cell type, the folliculostellate cell, the anterior pituitary can also synthesize numerous other non-classical peptides, growth factors, cytokines, binding proteins and neurotransmitters that are important for paracrine and/or autocrine control of anterior pituitary secretion and/or cell proliferation under defined physiological conditions.

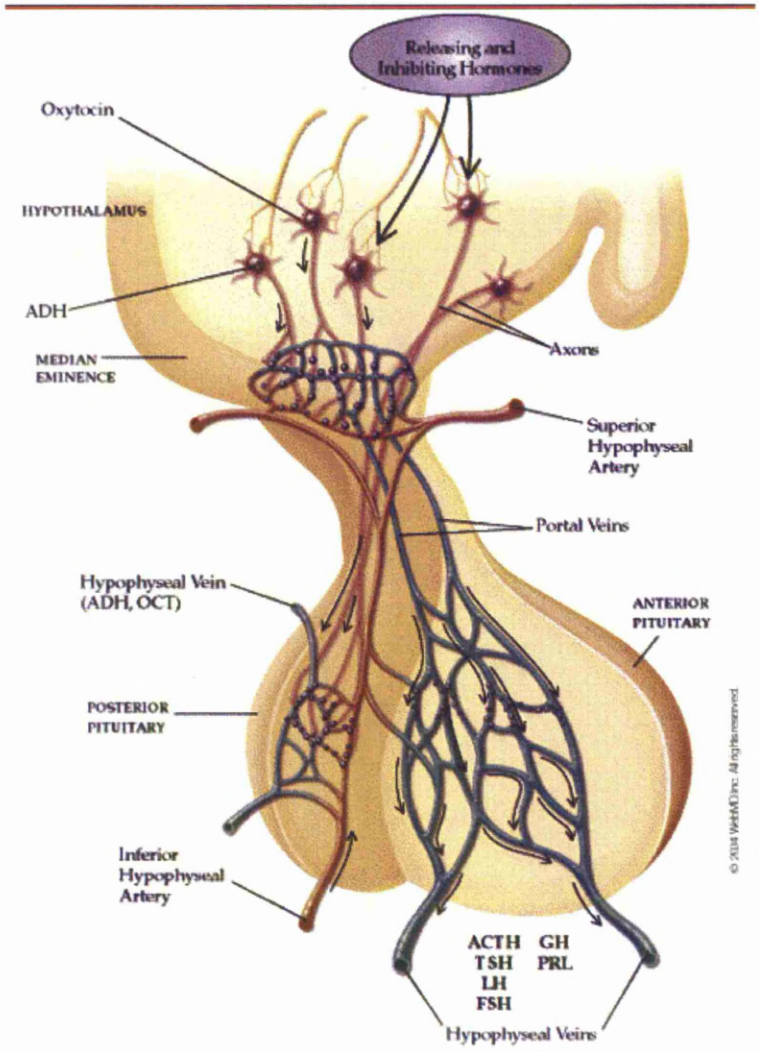


Figure 1.1: Schematic showing the vascular and hormonal function of the hypothalamus and pituitary gland (Source of image: www.medscape.com)

The anterior lobe produces the following hormones, which are regulated by the hypothalamus:

- GH - Stimulates growth of bone and tissue (growth hormone deficiency in children results in growth failure. Growth hormone deficiency in adults' results in problems in regulating body fat and muscle and bone mass resulting in increased body fat and reduced muscle and bone mass. It is also involved in emotional well-being.

- TSH - Stimulates the thyroid gland to produce thyroid hormones. Lack of thyroid hormones either because of a defect in the pituitary or the thyroid itself is called hypothyroidism.
- ACTH - Stimulates the adrenal gland to produce several related steroid hormones
- LH and FSH - Hormones that control sexual function and production of the sex steroids; estrogen and progesterone in females and testosterone in males
- Prolactin - Hormone that stimulates milk production in females

The posterior lobe produces the following hormones, which are not regulated by the hypothalamus:

- Antidiuretic hormone (vasopressin) - Controls water loss by the kidneys
- Oxytocin - Contracts the uterus during childbirth and stimulates milk production

The hormones secreted by the posterior pituitary are actually produced in the brain and carried to the pituitary gland through nerves and stored within vesicles in the pituitary gland.

Regulation of Pituitary Hormones

Secretion of anterior pituitary hormones is not autonomous. Each hormone is subject to regulation by hypothalamic peptides and, with the possible exception of prolactin, subject to the fundamental endocrine regulatory mechanism of negative feedback by the hormone from the target gland. This negative feedback control is at both the hypothalamic and pituitary levels and ensures precise homeostatic maintenance of physiologically appropriate hormonal secretion. If the primary gland fails, this results in reduced negative feedback and consequently increased hypothalamic and pituitary stimulation and secretion. Conversely, primary over-activity of the target gland results in increased negative feedback and diminished hypothalamic and/or pituitary stimulation. Pituitary hormones are

synthesized as part of large precursor molecules and they are then cleaved into fragments that are secreted into the circulation. One fragment is the main functional and the other co-secreted fragments have no known function as endocrine factors.

SECTION 2: GROWTH HORMONE

Growth hormone (GH) is synthesized by the acidophilic somatotroph cells of the anterior pituitary gland which make up 35% to 45% of pituitary cells. Human GH is a single chain protein with 191 amino acids and two disulfide bonds. The GH gene is located on chromosome 17. Approximately 75% is secreted in the 22kD form, while the remainder consists of a 20kD variant produced by alternate splicing. GH is secreted by the somatotroph cells located primarily in the lateral wings of the anterior pituitary. The morphological characteristics and number of these cells are remarkably constant throughout life, while secretion changes over time.

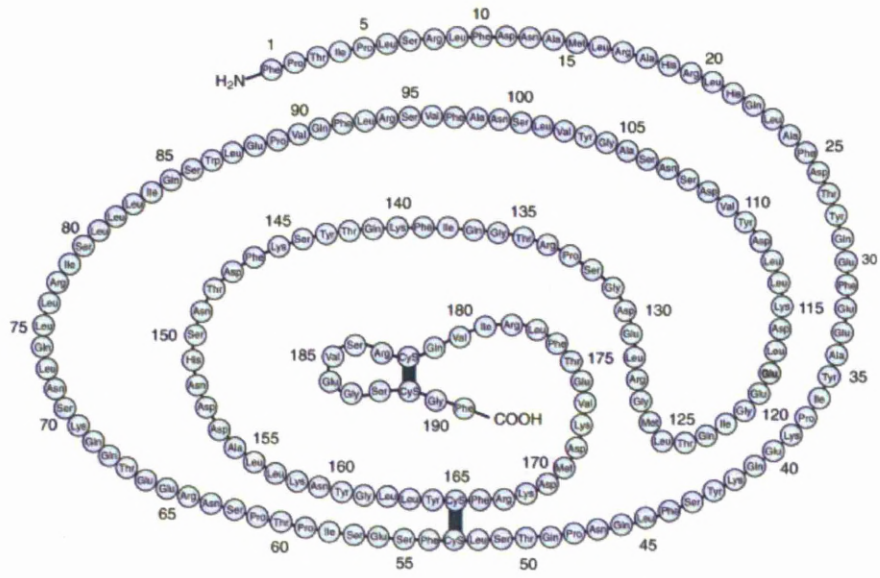


Figure 1.2: Covalent structure of the human growth hormone
(Source: Chawla RK, *et al* Ann Rev Med 1983;34:519-547)(8)

Pulsatile GH secretion in Humans

GH secretion occurs in a pulsatile fashion with a circadian rhythm in normal adults characterised by stable low levels interrupted by secretory bursts with a maximal release in the second half of the night. These GH-secretory bursts follow from multiple GHRH-secretory bursts into the hypophyseal portal circulation. In both children and young adults, maximal GH secretion occurs with the onset of the first slow wave sleep (usually within the first hour of sleep) (9). In adult men, the GH secretory pulse with the onset of slow wave sleep (phase 3 and 4 NREM sleep), is the highest and sometimes the only pulse observed throughout the day. In addition to this gender related difference in rhythmicity, the cumulative GH secretion in a 24-hour period is also influenced by age, pubertal status and nutritional status. Between pulses, GH levels are extremely low and in the majority of normal healthy adults, it is below the lower detection limit of assays; making random GH measurements unlikely to be of any value (apart from in states of excess GH secretion) (10).

In women with normal menstrual cycles, 24-hour GH pulses are higher than age matched men, daytime pulses are more frequent and the sleep associated GH pulse is not the major contributor of the total GH release (see figure 1.3). In a small group of healthy middle-aged men and premenopausal women, highly sensitive immunofluorometric assays (sensitivity 0.011 μg/l) and blood sampling at 10-min intervals showed the 24-h GH secretion was three times higher in the women, the GH secretory episodes were larger and more prolonged, more GH was secreted per burst, and maximal plasma GH concentrations were higher than in men (11).

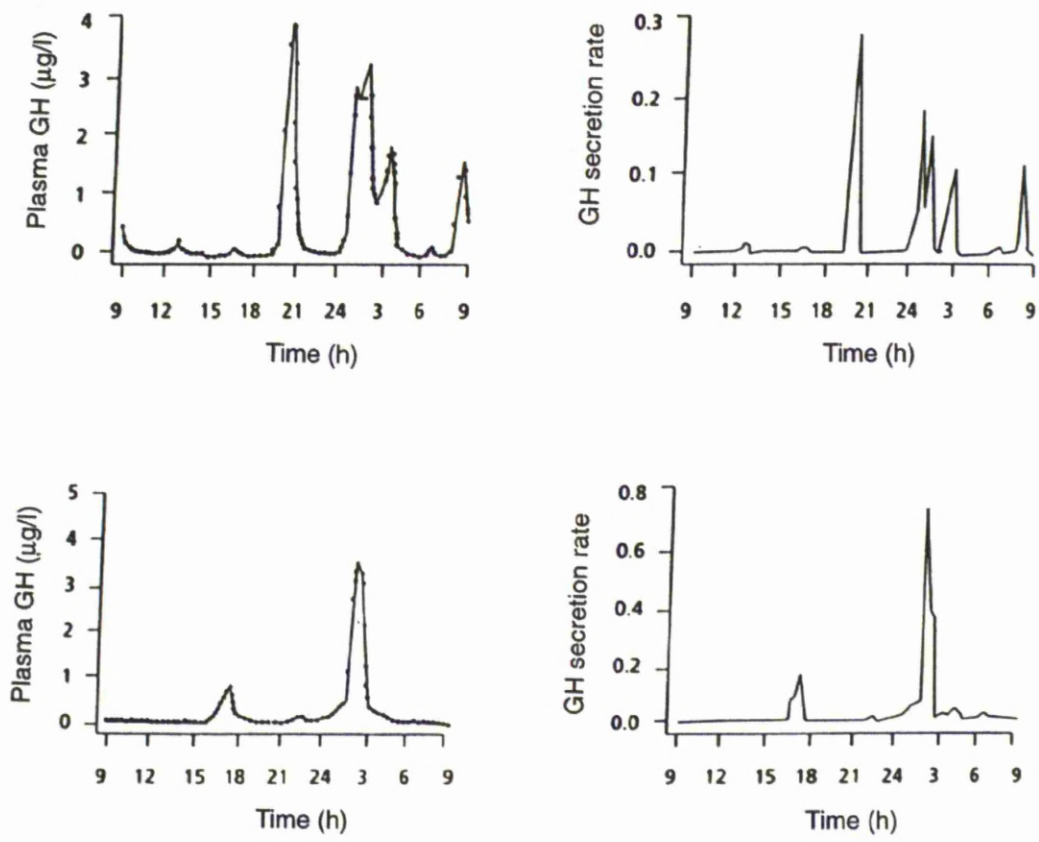


Figure 1.3: Profile of 24-hour serum GH in human female (top panels) and males (bottom panels) subjects (11).

A. REGULATION OF THE GROWTH HORMONE

Biologically active GH (GH1; OMIM 139250) binds its transmembrane receptor (GHR), which dimerizes to activate an intracellular signal transduction pathway leading to synthesis and secretion of insulin-like growth factor 1 (IGF-1; OMIM 147440). In plasma, IGF-1 binds to the soluble IGF-1 receptor (IGF-1R; OMIM 147370). At target cells, this complex activates signal-transduction pathways that result in the mitogenic and anabolic responses that lead to growth.

Two hypothalamic hormones regulate GH secretion; Growth Hormone Releasing Hormone (GHRH) with a stimulatory action at the level of gene transcription and somatostatin (SST) with an inhibitory effect on the GH secretion from the pituitary gland. Various synthetically produced GH releasing compounds and the recently discovered natural

hormone *Ghrelin* probably have a dual effect in increasing the release of GHRH and inhibiting SST action, thereby resulting in a very powerful stimulation of GH secretion. GH acts both directly through its own receptor and indirectly through the induced production of IGF-1. IGF-1 is synthesized both in the liver and in the periphery, and is an important mediator of GH actions. It circulates bound to a number of different binding proteins of which IGFBP-3 is the most important.

B. FACTORS CONTROLLING GROWTH HORMONE SECRETION

Hypothalamic factors

It is thought that the pulsatile release of GH is mediated via the alternating secretions of GHRH and SST (12); GHRH is released during the trough of SST secretion.

GHRH, synthesised in the arcuate nucleus and ventromedial nucleus, stimulates both GH synthesis and secretion. GHRH exists in two major forms in-vivo; a 40 amino acid and 44 amino acid form, however, only the first 29 amino acid residues are required to exert full biological activity (13). In humans, the gene encoding for GHRH is located on chromosome 20 (14). The effect is mediated by the human GHRH receptor, expressed in pituitary cells (15). The GHRH receptor is a 423 amino acid structure and the receptor gene has been mapped to chromosome 7 (16). In addition to the hypothalamus, GHRH synthesis has also been found at the cerebral cortex, brainstem, testis and placenta suggesting that GHRH has a role in the autocrine and paracrine regulation of extra-hypothalamic tissues (17).

SST was discovered in the late 1960's by Krulich et al (18). SST inhibits GH release without affecting synthesis (19, 20) and arises from the periventricular and paraventricular nuclei. SST exists in two main forms; 14 amino acid and a 28 amino acid peptide, although larger forms are known to exist (21). The hypothalamus contains large concentrations of

SST containing cells, however these cells are also found in the gastro-intestinal tract, particularly the secretory cells of the gut and pancreas (22). Five SST receptor subtypes have been characterized and the type 5 receptor (sst-5) appears to mediate most of the suppression of GH secretion by SST (23). In addition to inhibiting GH release, SST also inhibits release of TSH, a range of gastro-intestinal endocrine and exocrine functions and antagonises the release of hormones from a variety of endocrine secreting tumours (22).

GH exerts a negative feedback effect on its own secretion. Daily subcutaneous administration of exogenous GH for two to five days decreases the endogenous GH response to GHRH (24-26). This effect may be mediated by an increase in IGF-1 concentrations. Recent evidence suggests that GHRH and SST secreting neurons may interact with the hypothalamus. In vitro SST inhibits GHRH release (27) and GHRH stimulates SST release from perfused rat hypothalami (28). Such intra-hypothalamic interactions between these two peptides may contribute to the regulation of pulsatile GH release.

Neurotransmitters

A number of central neurotransmitters as well as a variety of peripheral feedback signals, regulate GH secretion either by acting directly on the anterior pituitary gland and/or by modulating GHRH or SST release, or both, from the hypothalamus (see figure 1.4). Pharmacological studies in humans reveal that activation of α_2 – adrenergic receptors and muscarinic cholinergic receptors stimulate GH secretion; antagonists of these receptors suppress GH release (29, 30). The influence of α_2–adrenergic neurons appears to be dominant since co-administration of clonidine (α_2 – adrenergic agonist) and atropine (a muscarinic cholinergic agonist) stimulate GH release. Furthermore, treatment with yohimbine (α_2 – adrenergic antagonist) can completely block the stimulatory effects on GH

secretion of enhancing cholinergic tone with pyridostigmine, a cholinesterase inhibitor (31).

In contrast, β-adrenergic receptors appear to mediate significant inhibitory effects on GH release (see table 1.1). Several studies have demonstrated that blockade of β-adrenergic receptors enhances the GH response to GHRH and other provocative stimuli but appear to have no effect on spontaneous GH secretion in boys with constitutional delay of growth (29, 30, 32). Administration of salbutamol, a β_2-adrenergic agonist, inhibits GH secretion whereas nicotinic cholinergic and α_1-adrenergic receptors appear to have lesser effects on GH secretion. Although α-adrenergic and cholinergic neurotransmission are likely to have important roles in regulating GH secretion in humans, it is still unknown whether the stimulatory effects on GH secretion of these pathways are mediated by suppression of SST release or stimulation of GHRH secretion or both.

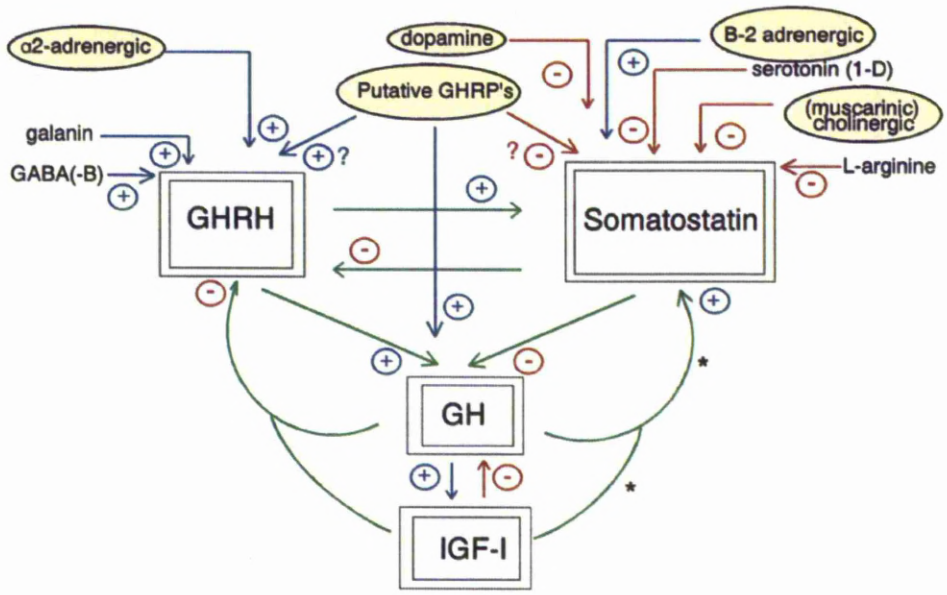


Figure 1.4. Principal neurotransmitters regulating GH secretion (30)

Sex Steroids

The relationship between GH release and sex steroids is complex. Circulating GH concentrations in males and females are similar before puberty but increase markedly at the onset of puberty (33). This increase in GH secretion during puberty is closely correlated to the increase in sex steroids (34, 35).

Although GH levels are three times higher in pre-menopausal women compared to age-matched men, the levels of IGF-1 in women are similar to men (11). The variation in GH secretion between sexes has been attributed to endogenous oestrogen with oestradiol levels being positively correlated with GH concentration and pulse amplitude (36). It is thought that the main gender difference is the amount of GH secreted per pulse, with higher pulse amplitude in women (11).

In women, the fall in oestradiol that occurs following the menopause is associated with a reduction in GH secretion (37, 38). In healthy post-menopausal women, a reduction in IGF-1 levels has been documented following oral oestrogen replacement (39). In contrast, when post-menopausal women were given transdermal oestrogen replacement, IGF-1 levels increased to pre-menopausal levels (39). The disparity between the two different routes of oestrogen administration on IGF-1 levels may be due to the first pass metabolism effect of oestrogen on the liver resulting in reduced hepatic IGF-1 production(39). The resultant decrease in IGF-1 therefore acts via a negative feedback mechanism to increase GH production in women. Furthermore, there is evidence to suggest that oestrogen also enhances the ability of GHRH to secrete GH (40, 41).

Female patients with growth hormone deficiency (GHD) have lower IGF-1 levels compared to men (42, 43) and they also require higher doses of GH to achieve similar clinical effects and IGF-1 levels to men (42, 43). Span et al also demonstrated that GHD

women on oral oestrogen replacement had lower IGF-1 levels compared to eugonadal GHD women at the same GH replacement dose (43).

Serum testosterone also stimulates GH secretion and during puberty, serum testosterone and GH levels are strongly correlated in boys (44). In a study of boys with hypogonadotrophic hypogonadism, testosterone treatment increases the amount of GH secretion per GH pulse. It is thought that the action of testosterone on GH secretion is mediated by the oestrogen receptors as the effect of testosterone on GH secretion is reduced by anti-oestrogens but not anti-androgens (45, 46) .

Thyroid Hormones

Studies in rodents have shown that thyroid hormones are able to stimulate GH release (47), whilst a reduction in GH secretion and levels were seen in rodents with hypothyroidism; this response was corrected with thyroid replacement (48). Thyroidectomy in neonatal rats also resulted in a reduction in pituitary GH mRNA levels (49). In humans, transient GHD in a patient with long-standing hypothyroidism, which was restored with thyroxine replacement, has also been reported(50). In hypothyroidism, IGF-1 and IGFBP-3 levels are diminished and this is possibly mediated through GH secretion (51, 52). In humans, experimental thyrotoxicosis factitia did not cause any significant change in these parameters.

Glucocorticoids

Glucocorticoids have two effects on GH secretion; chronic glucocorticoid exposure inhibits, whilst acute glucocorticoid administration enhances GH release and causes a transient increase in circulating GH (53). Casanueva et al (53) showed that plasma GH concentrations increasedtwo hours after administration of dexamethasone and peaked at

three hours, then decreased five hours post dose. The exact mode of action of glucocorticoids on GH secretion is unknown but the secretion of GH to various stimuli is blunted in patients on chronic glucocorticoid treatment and in those with Cushing's syndrome (54).

Dexamethasone can also induce a marked increase of serum IGF-1 and a minor increase of IGFBP-3 (55). Corresponding changes are found in Cushing's syndrome (56). This can be explained by stimulation of gene expression in the liver (57). Cortisol also suppresses the IGF-1 expression in osteoblasts (58).

Ghrelin

Ghrelin is a natural ligand of the growth hormone secretagogue receptor (GHS-R) (59) and is known to be a potent stimulator of GH release in healthy adults (60). The maximum stimulatory effect on GH secretion by ghrelin is ~ 5 fold greater than that achieved by GHRH. Ghrelin induced GH release requires an intact hypothalamic-pituitary pathway as shown by the proportionately lower GH levels stimulated by ghrelin *in vitro* compared to *in vivo* and in patients with organic disease of the hypothalamic pituitary region (61).

Although ghrelin stimulates GH secretion directly, a number of studies have demonstrated that ghrelin is not essential for GH secretion induced by well-known physiological parameters such as exercise, fasting and insulin induced hypoglycaemia (62, 63). Gut derived ghrelin, therefore, is not a major regulator of pituitary GH secretion under normal conditions.

Other factors influencing GH secretion

Stress, hypoglycaemia and ingestion of protein (high levels of circulating amino acids) stimulate GH secretion, while high levels of glucose and free fatty acids (FFA) inhibit secretion. With the introduction of reliable radio-immunological assays it was recognized

that circulating GH was blunted in obese subjects, and that normal aging was accompanied by a gradual decline in GH levels. The latter observation led Rudman et al. to the hypothesis that many of the senescent changes in body composition and organ function were related to or caused by hypo-somatotropinaemia (64) which is termed "somatopause". More recent studies have documented that hypopituitary adults with severe GHD are characterized by increased fat mass and reduced LBM (65). It is also known that normal GH levels can be restored in obese subjects following massive weight loss (66), and that GH substitution in GHD adults normalizes body composition (65).

Table 1.1: Summary of factors that control GH release in humans

Factors	Stimulatory	Inhibitory
Physiologic	Fasting	Post prandial hyperglycaemia
	Exercise	Elevated NEFA
	Slow wave sleep	Aging
	Stress	
Hormones	Insulin	Glucocorticoids – Chronic
	Sex steroids	
	Glucocorticoids – Acute	
	Ghrelin	
	Thyroid hormones	
Neuropeptides	GH-releasing hormone (GHRH)	Somatostatin (SS)
	Atropine (muscarinic cholinergic agonist)	Salbutamol (β_2 - agonist)
	Clonidine (α_2 - adrenergic agonist)	Yohimbine (α_2 - antagonist)
	β-adrenergic antagonist	Nicotinic cholinergic agonists
	Serotonin agonists	Serotonin antagonists
	Pyridostigmine	
	Growth hormone secretagogues	
	Galanin	
	Hexarelin	
	Dopamine	

C: METABOLISM OF GROWTH HORMONE

In health, the growth hormone receptor (GHR) is the principal regulator of sensitivity to growth hormone (GH).

Biologically active growth hormone binds its transmembrane receptor, GHR which dimerizes to activate an intracellular signal transduction pathway leading to synthesis and secretion of IGF-1. In plasma, IGF-1 binds to the soluble IGF-1 receptor. At target cells, this complex activates signal-transduction pathways that result in the mitogenic and anabolic responses that lead to growth.

Growth hormone receptors have been identified in many tissues including muscle, adipose tissue, liver, heart, kidney, brain and pancreas (67, 68) and are part of the cytokine receptor superfamily (69). The GHR exists in three forms, the full-length, long form composed of 620 amino acids, and two short forms of either 277 or 279 amino acids in length (70). One of the short forms is membrane-bound, with a much shorter cytoplasmic domain. The soluble form of the GHR is also known as the GH-binding protein (GHBP) (71), which is identical to the extracellular domain of the GHR.

The initial step in the activation of the GH signal transduction pathway is dimerization of the receptor (72). Janus kinase 2 (JAK2) is the receptor-associated kinase inducing tyrosine phosphorylation (73). Dimerization brings together the two JAK2 molecules, and the activated JAK2 phosphorylates certain tyrosine residues of the cytoplasmic domain of the receptor, and other molecules adjacent to the receptor-JAK2 complex. Among these are the signal transducers and activators of transcription (STAT) proteins (74). The phosphorylated STAT proteins translocate into the nucleus, bind to DNA, and activate the transcription of specific genes. The administration of GH in vivo to hypophysectomized rats induces stimulation of STAT 1, -3, -5 in liver nuclear fraction (75-77), whereas this effect has been shown in non-hypophysectomized rats for JAK2 and STAT 5 (78). GH

activates STAT 5 proteins in adipocytes (79) and a mutation in the gene for STAT 5b results in IGF-1 deficiency and GH insensitivity with profound growth failure (80). The JAK/STAT pathway is, in turn, negatively regulated by several proteins, including suppressor of cytokine signalling (SOCS) 1–7 (70). Analysis of STAT5b deficient mice shows that they have a gender related deficiency in body growth, which is seen in males but not females. They also had elevated plasma GH levels and reduced IGF-1 as well as reduced GH induced lipolysis in adipose tissue. In adipocytes, fibroblasts and Chinese hamster ovary cells the insulin-responsive docking proteins, insulin receptor substrate (IRS)-1 and -2, are rapidly phosphorylated in response to GH, and phosphorylated tyrosine residues in these docking proteins provide binding sites for phosphatidyl-inositol-3′-kinase (PI-3-kinase)(81). GH also affects adaptor proteins leading to the activation of the Ras/mitogen-activated protein (MAP) kinase (82) which might involve cross-talk with the activated epidermal growth factor (EGF) receptor (83). Protein kinase C also appears to play a role in GH signalling, as treatment with inhibitors of protein kinase C decreases GH induction (70).

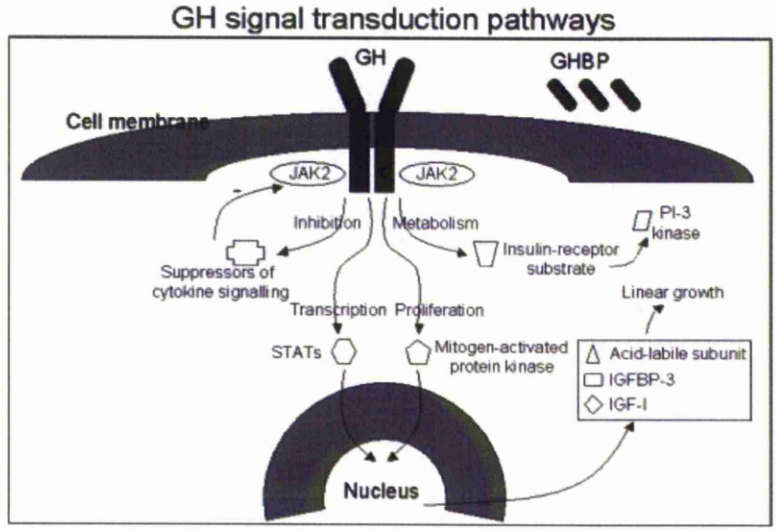


Figure 1.5: GH signal transduction pathway
[Source: Adapted from N Eng J Med, 2003 349(12)](84)

Insensitivity to Growth Hormone

Animal studies and human mutational analysis have both supported a central role for the IGF system in postnatal growth. Responsiveness to GH in target cells is primarily dependent upon the expression of the GH receptor.(84)

In the normal GH-IGF axis, there are potential biochemical defects capable of causing insensitivity to GH: abnormalities of the GHR or of the binding protein or both and abnormal signal transduction. Genetic mutations leading to biochemical defects in this axis usually present in children as short stature and often result in complex syndromes.

Heterogeneous mutations which may result in normal growth but later impact on response to therapy with rhGH are largely unexplored with the exception of exon 3 which encodes the extracellular domain of the GHR gene.

D. FUNCTIONS OF GROWTH HORMONE

A critical concept in understanding growth hormone activity is that it has two distinct types of effects:

- Direct effects are the result of growth hormone binding its receptor on target cells. Adipocytes, for example, have growth hormone receptors, and growth hormone stimulates them to break down triglyceride and suppresses their ability to take up and accumulate circulating lipids.
- Indirect effects are mediated primarily by IGF-1, a hormone that is secreted from the liver and other tissues in response to growth hormone. The majority of the growth promoting effects of growth hormone is actually due to IGF-1 acting on its target cells.

Growth Hormone on Growth

Growth is a very complex process, and requires the coordinated action of several hormones. The major role of GH in stimulating body growth is to stimulate the liver and other tissues to secrete IGF-1. IGF-1 stimulates proliferation of chondrocytes (cartilage cells), resulting in bone growth. Growth hormone also seems to have a direct effect on bone growth in stimulating differentiation of chondrocytes(85).

IGF-1 also appears to be the key player in muscle growth. It stimulates both the differentiation and proliferation of myoblasts. It also stimulates amino acid uptake and protein synthesis in muscle and other tissues.

Metabolic functions of Growth Hormone

Growth hormone has important effects on protein, lipid and carbohydrate metabolism. In some cases, a direct effect of GH has been clearly demonstrated, in others, IGF-1 is thought to be the critical mediator and in some cases it appears that both direct and indirect effects are at play.

- Protein metabolism: In general, GH stimulates protein anabolism in many tissues. This effect reflects increased amino acid uptake, increased protein synthesis and decreased oxidation of proteins.
- Fat metabolism: Growth hormone enhances the utilization of fat by stimulating triglyceride breakdown and oxidation in adipocytes.

- Carbohydrate metabolism: Growth hormone is one of a battery of hormones that serves to maintain blood glucose within a normal range. Growth hormone is often said to have anti-insulin activity, because it suppresses the abilities of insulin to stimulate uptake of glucose in peripheral tissues and enhance glucose synthesis in the liver. Somewhat paradoxically, administration of GH stimulates insulin secretion, leading to hyperinsulinemia.

SECTION 3: GROWTH HORMONE DEFICIENCY

Two main categories for GHD are childhood onset GHD (COGHD) which maybe congenital or acquired and adult onset GHD (AOGHD) which may have been developed childhood or be acquired in adult life.

A: Childhood Onset GHD

Idiopathic GHD is the most common cause of GHD in children(86). It is a poorly defined and often reversible condition that presents with short stature and low growth velocity for age with a prevalence of 1 in 3500 children. Most cases of COGHD are idiopathic (87) and are not necessarily associated with other pituitary hormone deficiencies (Table 1.2). Various genetic factors have been found to be responsible for idiopathic GHD.

GHD in childhood is also associated with congenital anomalies and malformations and there are many acquired causes (Table 1.2). There are several forms of congenital GHD, which are subdivided into idiopathic, genetically determined and structural lesions of the hypothalamic-pituitary area. There have been several large studies examining the aetiology of GHD in children. A previous survey of 325 children with GHD showed that 21.9% had organic lesions, 69.5% were idiopathic and 8.6% had hereditary forms of GHD (88). A later survey in England and Wales in 1977 also showed similar results in children with GHD, with organic lesions accounting for a quarter to a third of cases, 3% were hereditary

and the remainder were idiopathic in nature (21). In children with idiopathic congenital GHD, the main abnormality is thought to lie in the hypothalamus, as the majority of these children are able to produce GH in response to GHRH or one of the GH releasing analogues (89).

It is thought that far more cases of GHD have a genetic aetiology than are currently recognised (88). GHD is often not absolute; there is a wide range of GH secretory capacity; from the mild to the severe end of the spectrum (90). Different genetic forms of GHD have been identified; some causing isolated GHD and others are associated with other pituitary hormone deficiencies.

Idiopathic GHD has been associated with birth trauma as a higher incidence of birth trauma (65%) is seen in those with hypopituitary dwarfism when compared to the normal population (3-4%) (88). Non-cephalic deliveries were also more commonly seen in patients with so-called 'idiopathic' GHD with higher incidences of breech and forceps deliveries in those with multiple hormone deficiencies compared with those with isolated GHD(21). The association with non-cephalic deliveries may be due to the high number of brain injuries resulting from such procedures, but it is also thought that idiopathic GHD may be a multi-factorial disorder(21). With improvements in obstetric practice, such lesions are now less common.

Vaginal bleeding occurring during the first two trimesters of pregnancy may also be another risk factor; 7.1% of pregnancies resulting in GHD were associated with vaginal bleeding compared with 3% in the normal population(21).

Table 1.2: Causes of Childhood Onset GHD

1. **Congenital Conditions**

    a. Anatomical anomalies
        i. Stalk dysgenesis, with or without ectopic posterior pituitary
        ii. Encephalocoele
        iii. Empty sella
    b. Genetic factors
        i. Mutations in the Pit-1 gene
        ii. Mutations in the PROP-1 gene
        iii. Mutations in the GHRH-receptor gene
        iv. Fanconi's syndrome
        v. Septo-optic dysplasia
        vi. Panhypopituitarism
    c. Idiopathic (usually due to GHRH deficiency)

2. **Acquired Conditions**

    a. CNS Tumours
        i. Hypothalamic
        ii. Pineal
        iii. Pituitary tumours
        iv. Craniopharyngiomas
        v. Rathke's cleft cysts

    b. Cranial Irradiation
        i. Administered for tumours or infiltrative diseases

    c. Infiltrative Diseases
        i. Langerhans' cell histiocytosis
        ii. Sarcoidosis
        iii. Tuberculosis
        iv. Lymphocytic hypophysitis
        v. Haemochromatosis

    d. Trauma

    e. Hypoxic Insult

B: Adult Onset GHD

In contrast to COGHD, adult onset GHD generally presents as part of a combined pituitary hormone deficiency phenotype (hypopituitarism) and is commonly attributable to a pituitary tumour or other structural lesions of the hypothalamic-pituitary axis (91, 92) and/or treatment with surgery or radiotherapy(93).

AOGHD commonly results from intra-cranial tumours, either as a direct consequence of the tumour or its treatment particularly after radiotherapy (94). The majority of these tumours are benign with pituitary adenomas being the main cause of hypopituitarism. In a study of 333 adult patients with hypopituitarism, pituitary adenoma was identified in 223 patients (91). Intra-cranial developmental tumours such as astrocytomas, medulloblastomas and craniopharyngiomas are much less common. These are developmental tumours and usually occur above the sella (88). Inflammatory granulomatous conditions affecting the pituitary may also lead to GHD (Table 1.3).

In another study of 165 patients with pituitary tumours when evaluated before surgery, 50 % already had evidence of GHD (95). After surgery about 80 % had evidence of GHD and it developed in all those who had post-operative radiotherapy. The incidence of AOGHD is not known, but indirect estimates based on the incidence of pituitary tumours suggest an incidence of 10 people/million annually (96) with a prevalence of probably about 1 per 10,000 in the European population (97).

In the development of hypopituitarism due pituitary tumours, loss of the hormones follows a characteristic sequence. GH secretion is the first one to be impaired followed by LH and FSH and finally TSH and ACTH.

Table 1.3: Causes of Adult Onset GHD

- Pituitary Adenoma
- Rathke's cysts
- Cranial irradiation
- Post-hypothalamo-pituitary surgery
- Post pituitary radiotherapy
- Para-sellar meningiomas
- Suprasellar germ cell tumours
- Post-partum pituitary necrosis
- Rathke's cysts
- Craniopharyngioma
- Metastatic disease in the pituitary gland
- Sub-Arachnoid Haemorrhage
- Lymphocytic hypophysitis
- Langerhans' cell histiocytosis
- Granulomatous infiltrations - Sarcoidosis, Haemochromatosis
- Idiopathic
- Traumatic Brain Injury
- Metastatic disease in the pituitary gland

GHD associated with pituitary tumours

Macroadenomas (pituitary tumours greater than 1 cm) are associated with one or more trophic hormone deficit in about 30% of cases(98) with direct compression and destruction of the surrounding normal pituitary leading to hormone hyposecretion. Other postulated mechanisms include primary mass effect of the tumour on the vascular portal system/pituitary stalk, raised intrasellar pressure affecting portal circulation, and focal pituitary necrosis secondary to prolonged interruption of portal blood supply(99, 100). While microadenomas (< 1 cm) rarely affect pituitary function(100), prolactin producing microadenomas often present with hypogonadism because of the suppressive action of a high prolactin (PRL) level on secretion of gonadotrophins (follicle stimulating hormone FSH and luteinising hormone LH). Among the peri-pituitary tumours causing central endocrine dysfunction, craniopharyngioma is the commonest. Craniopharyngioma is the most common tumour in the hypothalamo-pituitary region to cause pituitary deficiency in childhood. The tumour usually arises from remnants of Rathke's pouch, an invagination of the epithelium within the third pharyngeal pouch from which the anterior pituitary evolves. Although histologically a benign tumour, craniopharyngiomas can be locally invasive, involving adjacent structures especially the optic tracts and base of the third ventricle. It usually has a solid and cystic component that may contain a cholesterol-rich fluid. GH deficiency (72%) is the most common endocrine abnormality at clinical presentation, followed by short-stature in 53% whereas ACTH, TSH and ADH deficiencies were found in approximately 25% of cases. Meningiomas disrupt the neuro-anatomic connections and vascular supply of the hypothalamic-pituitary axis to result in hypopituitarism either directly from mass effects or as a result of treatment(s) (101).

GHD following Surgery

Hypopituitarism can be a consequence of pituitary surgery. The incidence and degree of hypopituitarism depend on several factors, including the size of the original tumour, the degree of infiltration, and the experience of the surgeon. Following surgery endocrine deficiencies of ACTH, TSH, GH, LH, FSH and ADH are highly likely, therefore these patients should be carefully monitored and appropriate hormonal replacement therapies commenced promptly.

Surgery for pituitary adenomas may also be followed by significant recovery of pituitary function; up to 50% of patients recover at least one pituitary hormone that had been deficient before trans-sphenoidal surgery (99, 102, 103). Postoperative improvement is more likely if there is no tumour on postoperative imaging and no neurosurgical or pathological evidence of an invasive tumour (102). GH is less likely to recover than gonadotrophins, ACTH and TSH (99). There is evidence that among those patients in whom recovery of pituitary function occurs, the process begins immediately after surgery (103).

GHD following radiotherapy

Growth hormone deficiency (GHD) is the most frequent complication and usually the only overt manifestation of neuroendocrine injury in the vast majority of irradiated patients including non-pituitary brain tumours.(104) Radiation-induced damage of the hypothalamo-pituitary axis (HPA) is believed to be neuronal rather than vascular(105). The hypothalamus is a highly radiosensitive structure and is thought to be the primary site of radiation damage, particularly with radiation doses of more than 50 Gy. The severity and speed of onset of radiation-induced GH deficiency is dose-dependent. Hypothalamic–pituitary dysfunction secondary to radiation is also time-dependent, with both increased

incidence and severity of hormonal deficits occurring with longer post-irradiation follow-up intervals (106-108). The progressive nature of the hormonal deficits following radiation damage to the HPA can be attributed to secondary pituitary atrophy consequent upon lack of hypothalamic releasing/trophic factors (109, 110) or delayed direct effects of radiotherapy on the pituitary.

GHD following Brain Injury and Subarachnoid Haemorrhage

Recent reports suggest hypopituitarism can follow traumatic brain injury (TBI) and sub-arachnoid haemorrhage (SAH) (101). It is estimated that the prevalence of anterior pituitary dysfunction following TBI might be as much as 30-70% (111) with a review by Schneider *et al* in 2007 showing up to 47% after SAH(112). Controversy exists as to the prevalence of hypopituitarism following SAH(113, 114) with a number of endocrine societies recommending that all patients should undergo evaluation of anterior pituitary function after SAH(115, 116).

Patients with an initial Glasgow Coma Scale of $\leq$ 13 should be evaluated for pituitary dysfunction although there is as yet no consensus for the earliest time when this evaluation should be carried out. GH is the first hormone to be deficient in 9-40% of patients following TBI (117). The natural course of changes in pituitary function in such patients is unclear. A prospective review of SAH survivors in the same Walton Centre for Neurology and Neuroscience as this study showed a prevalence of of hypopituitarism of 12% after 12 months follow-up with no predictive factors for the development of hypopituitarism(118). In the study, Gardner et al recommended the screening for the presence of hypopituitarism at least 12 months after the SAH, when potentially reversible pituitary dysfunction has resolved.

Early post traumatic pan hypopituitarism however generally persists as a permanent feature and will require appropriate hormone replacement therapy (111).

C: DIAGNOSIS OF GROWTH HORMONE DEFICIENCY

Diagnosis of Childhood Onset GHD

The diagnosis of anterior pituitary GH deficiency in children is difficult due to the pulsatile nature of GH secretion. Various tests are available to test GH reserve and can be divided into provocation tests and those measuring spontaneous GH secretion. As a normal child may fail a single provocative test, it is recommended that two abnormal provocative tests are required to diagnose GHD (119). In order to distinguish between genuine GHD and constitutional delay in growth and puberty, oestrogen or androgen should be administered for a few days before a GH provocative test (120-122).

COGHD requires reconfirmation in adulthood as 35% of idiopathic COGHD patients showed normal GH secretory responses when retested as adults (123). This may be related to maturation of the hypothalamic-somatotroph axis.

Diagnosis of Adult Onset GHD

In the last two decades, with the increased recognition of GHD as a distinct clinical entity in adults and the greater availability of growth hormone replacement therapy, it has become increasingly important to accurately diagnose adults with symptomatic GH deficiency. Adult GHD (AGHD) is a syndrome consisting of a cluster of clinical features such as truncal obesity (124), reduced muscle strength and increased fatigability (125) and decreased psychological well-being which has been demonstrated by Wallymahmed *et al* in similar patient population in the North West of England (126). Although these clinical features are vague and non-discriminatory, GHD may be suspected if there is also a history of pituitary tumour, surgery or radiotherapy.

The diagnosis of severe GHD is straightforward biochemically within the appropriate clinical context. In patients with hypothalamic–pituitary disease, the syndrome of adult GHD characteristically manifests with derangements in body composition, physical, and psychological function (116). Partial GHD is at present not a well-defined clinical entity in adults but the presence of a low IGF-1 concentration increases the likelihood of GHD. Inconclusive testing should be followed by ongoing clinical evaluation and repeat testing. The 2007 Consensus guidelines for the diagnosis and treatment of adults with GH deficiency II (116) state that patients who should be tested for GHD are those who show evidence of hypothalamic–pituitary disease, and in whom there is an intention to treat. This includes patients from the following three groups:

(1) Those with signs and symptoms of hypothalamic–pituitary disease (endocrine, structural, and/or genetic causes)

(2) Those who have received cranial irradiation or tumour treatment and

(3) Those with traumatic brain injury (TBI) or subarachnoid haemorrhage (116).

One stimulation test is sufficient for the diagnosis of adult GHD. Not all patients suspected of having GHD however, require a GH stimulation test for diagnosis. Patients with three or more pituitary hormone deficiencies and an IGF-I level below the reference range have a >97% chance of being GHD, and therefore do not need a GH stimulation test.

D: GROWTH HORMONE STIMULATION TESTS

Insulin tolerance test

The insulin tolerance test (ITT) is the accepted 'gold standard' for diagnosing GHD(127). The ITT is not without problems and should only be used in experienced units and is contraindicated in patients with cardiovascular, cerebrovascular disease, epilepsy, severe

untreated hypoadrenalism and the elderly (127-129). However, in experienced endocrine units, the ITT has been reported to be safe (130).

Intravenous insulin is administered to reduce plasma glucose to below 2.2mmol/l and blood samples taken every 30 minutes for 2 hours to measure GH and cortisol. The peak concentration of GH usually occurs 30-60 minutes after symptomatic hypoglycaemia occurs. Using the ITT, the cut-off for severe GHD is a peak concentration of < 3μg/L (9mU/L) (127).

The ITT should be considered only in the appropriate clinical context, i.e., in patients with evidence of hypothalamic disease, following pituitary surgery or irradiation and in those with childhood onset GHD(127). Furthermore these patients are likely to have multiple pituitary hormonal deficits and the probability of GHD increases with increasing numbers of pituitary hormonal deficiencies (92). Patients undergoing the ITT should be receiving stable and adequate doses of other hormone replacement therapy(127).

Glucagon Stimulation Test

The advantage of the glucagon stimulation test (GST) is that, like the ITT, it is able to assess both cortisol and GH secretion(129, 130), also there is good correlation between the ITT and GST in releasing cortisol and GH (131-133). With regards to GH release, the GST has also been shown to be more effective than arginine(132). The GST is thought to have similar specificity and sensitivity to the ITT at the 9mU/L (3 ug/L) cut-off (132, 134). Furthermore a later study comparing the different GH secretogoues showed that the mean peak GH response was similar between the GST and ITT and that the GST was better than the clonidine, pyridostigmine and galanin tests (135). The GST has been shown to be second only in terms of efficacy to the ITT in the stimulation of GH and ACTH release (128, 132) and can therefore be considered as a suitable alternative when the ITT is contraindicated. Unlike the ITT there are few contra-indications to the use of GST.

The mechanism of glucagon induced GH release is unclear. However, several studies have suggested a relationship between the falling blood glucose levels later in the test and peak GH levels (136-138). It is likely that other mechanisms other than falling blood glucose levels are involved as the hyperglycaemia found in diabetic patients does not seem to impair the glucagon induced GH release (131, 139).

Another possible mechanism for glucagon induced GH release is via noradrenaline secretion. Glucagon has been shown to induce noradrenaline release in healthy volunteers (140). Two peaks of noradrenaline release have been identified; 30 minutes and 150 minutes post glucagon administration. It was also demonstrated that the timing of side-effects corresponded to the second noradrenaline peak and peaks in GH and cortical (140). However, there was no correlation between the severity of the side-effects and hormonal levels reached. The increase in plasma noradrenaline may be responsible for stimulating GH release possibly via α-receptors as administration of a β-blocker enhances GH secretion (133, 137, 141).

Intravenous glucagon causes less GH release compared to both intramuscular and subcutaneous glucagon (142, 143) and the intramuscular route has been suggested as being more reliable and effective at releasing GH(144).

The peak GH levels obtained with the ITT and GST have been shown to be similar in both patients and healthy subjects(135, 138) and there is good correlation between the two tests ($r = 0{\cdot}645$) in peak GH levels obtained (131). Two studies comparing the GST and ITT in healthy subjects have shown the GST to be as potent as the ITT in releasing GH in healthy individuals (135, 145). Another study showed that when propanolol is taken 2 h before glucagon administration greater GH release is obtained with the GST compared to the ITT(133).

Contra-indications to the GST include presence of an underlying insulinoma and phaeochromocytoma (146). An audit of over 500 consecutive GSTs in patients with hypothalamic-pituitary disease showed that the main side effects were nausea, sweating or headache, occurring during 20 % of the tests (147). The glucagon stimulation test has proved to be a safe, low-cost and effective means of stimulating GH secretion; it does not require the constant supervision of an experienced doctor and therefore can be considered as a suitable alternative to the ITT.

Other stimulation tests

Many tests have been described to assess GH secretion. Newer tests such as the arginine and growth hormone releasing hormone (GHRH) and pyridostigmine and GHRH tests have been used as alternatives for the diagnosis of GHD (148, 149). The arginine (which inhibits somatostatin release) and GHRH test is considered to be a suitable alternative to the ITT, and its ability to stimulate GH release is unaffected by the age of the subject(148). The pyridostigmine and GHRH test has been shown only to be reliable for those between the ages of 20-40 years (148). However, a later study comparing the pyridostigmine and GHRH test to the ITT showed the pyridostigmine and GHRH test to be a more potent GH secretogogue than the ITT (149). In addition to the new tests, the arginine and glucagon tests can be used as alternatives to the ITT for patients with contra-indications to the ITT [Growth Hormone Research Society 1998] (127). Other agents used as secretagogues have included L-dopa, nocturnal sleep, clonidine, hexarelin (150) and exercise (151).

Overall, recent evidence from Toogood *et al* in 2012 shows that the ITT, AST, and GST produce similar GH peaks, are influenced by similar clinical factors, and identify patients with similar features of GH deficiency at a diagnostic threshold of 3 μg/L (152).

Measurement of spontaneous GH secretion

GH secretion is episodic in nature and therefore a single GH measurement is of limited value in diagnosing GHD. Plasma GH is measured by RIA (polyclonal or monoclonal) or by IRMA (immunoradiometric -dual monoclonal). The measured GH concentration therefore depends on the type of assay used in its detection. This is because endogenously secreted GH has different isoforms (22- and 20-kd monomers, acetylated 22K) with different antibodies binding to a heterogeneous spectrum of GH isoforms. Also, the secretion of these differing isoforms has an inter-individual variability, making it difficult to compare GH results from different immunoassays(153). 24 hour repeated serum GH measurements have been used but these are time-consuming; require hospital admission and the results are disappointing. There is considerable overlap between GHD patients and healthy subjects, so it is not a reliable test (124). Urinary GH measurement in conjunction with insulin-like growth factor-1 has been advocated but is unreliable due to GH concentrations varying significantly between individuals and within individuals from day to day (154).

E: CLINICAL FEATURES OF GROWTH HORMONE DEFICIENCY

Whilst diagnosis of GH deficiency in children is straightforward in its presentation with growth retardation, diagnosis of adult onset GH deficiency is much more challenging; however, in the presence of a probable cause and other hormonal deficiencies, GHD can be attributed as causative of such symptoms.

There is no single symptom or sign that is pathognomonic of GH deficiency in adult life, but a well-defined constellation of symptoms and signs has been identified in adults with GH deficiency, leading to the recognition of a "growth hormone deficiency syndrome". In

adult life, GH deficiency is associated with altered body composition, altered metabolism, reduced exercise capacity, and impaired quality of life (93, 155).

Effect of GHD on Quality of Life

One of the best recognised clinical aspects of adult growth hormone deficiency (AGHD) syndrome in hypopituitary adults is reduced psychological well-being. Hypopituitary patients with GHD describe a number of adverse features such as reduced vitality and energy, greater emotional lability, social isolation, low mood and an overall poor life quality (156, 157). Initial objective measurements of QoL were done using generic health status questionnaires such as Nottingham Health Profile (NHP) and Psychological General Well Being Schedule (PGWB) (156, 157), but more recently "disease-specific" questionnaires have been more widely used (158). The Assessment of Growth Hormone Deficiency in Adults questionnaire (QoL-AGHDA/AGHDA) (159) is well validated and normative data is available for UK, Spanish and Swedish populations. The AGHDA questionnaire has been criticised for the lack of normative reference data in different countries; for its inability to take into account differing QoL expectations in different countries and by its inability to separate the measured QoL into domains. Another questionnaire for use in AGHD is the newer QLS-H (Quality of Life Satisfaction-Hypopituitarism) questionnaire, which has the advantage of providing scores which are first weighted by each individual patient according to the importance they place on a particular item. The National Instititute for Health and Clinical Excellence (NICE) recommend treatment for AGHD based on an impaired QoL-AGHDA questionnaire score and it is used for the purpose of this study. The QLS-H questionnaire has also been validated and widely translated (160, 161). The impairment in life quality reported by adults with GHD is heterogeneous in that even with severe biochemical GHD, some adults

report a normal QoL compared to others with less severe GH deficiency(162). Also, adults with COGHD have less significant impairments in life quality in comparison to those with who develop GH deficiency in adulthood. The explanation suggested for this difference is probably due to an adaptation mechanism whereby those with COGHD have adapted to less demanding challenges in life which they can cope with and which is within their limitations (162, 163). Li Voon Chong *et al.* (164) demonstrated that when one considers the effect of normal aging process with QoL in patients with organic hypothalamic pituitary disease, elderly patients (mean age 71 years) with GHD were shown to have significant impairments in multiple aspects of QoL (e.g. emotional reaction, social isolation, mental fatigue) compared to matched controls. Interestingly, Li Voon Chong *et al* and Wallymahmed *et al* studying GHD adults in the same endocrine service as those studied in this research project showed the overall scores for QoL impairments in the elderly were less severe when compared to younger GHD adults (164, 165). Also when the same cohort with untreated GHD was followed up over a further 2 years, there were continued impairments in some QoL domains (e.g. less energy, less vitality, lower self-esteem) over the period of follow up. Additionally, the control group also had similar impairments in life quality to a degree which was indistinguishable from GHD patients at the end of 2 years (166).

The mechanism(s) underlying the impairment in life quality in GHD adults is possibly multifactorial and seems to bear no correlation to metabolic, body composition or biochemical parameters. Adults with hypothalamic-pituitary disease require life-long hormone replacement treatment(s) and follow-up. It is therefore conceivable that the combination of having a chronic illness requiring long term care, the effect of having had neurosurgery and/or radiotherapy and the prospect of possibly requiring these in future and the effects of treatments on vision, neurological and psychological function may all put

together contribute to the development of significant impairments of QoL in adults with hypopituitarism and GHD (161, 165, 167).

Effects of GHD on body composition

The most conspicuous feature of adult GH deficiency is an abnormal body composition characterized by increased fat mass and reduced lean body mass (LBM) (164, 168-171). Body fat accumulates preferentially in the visceral and upper body subcutaneous compartment and may cause adverse metabolic effects such as impaired insulin sensitivity (172, 173). In addition, these patients have reduced bone mass compared with matched healthy controls, and are at increased risk of sustaining osteoporotic fractures(174).

The body composition of young and middle-aged adults who develop GHD after puberty differs from healthy age matched controls with an increase in total body fat mass and central obesity (126, 169, 175-179). Evidence for a central fat distribution comes from assessment of waist to hip ratio, skin fold thickness, anthropometric measurements, bioelectric impedence (180) and computerised tomography studies (176, 180). It seems that the lack of GH causes the redistribution of fat from peripheral to central depots. Patients with GHD tend to be heavier, have lower lean body mass and total body water (181).

GH replacement has been shown to correct the abnormal fat distribution in patients with GHD. GH replacement in adults with GHD has been shown to normalise body composition by leading to an increase in lean body mass and a decrease in fat mass (158, 175-177, 182-186). This change in lean body mass (LBM) and fat mass occurs in both COGHD and AOGHD (162) and is maintained for at least 7-10 years (187, 188). It is also known that normal GH levels can be restored in obese subjects following massive weight loss(66).

AGHD and hypopituitarism have also been associated with a number of risk factors for cardiovascular disease, including vascular endothelial dysfunction, dyslipidaemia, and insulin resistance(189). Reduced left ventricular mass, impaired cardiac systolic function,

and impaired response to peak exercise have also been reported(190). These findings, along with the reduced lean body mass, result in reduced muscle strength and exercise capacity.

It is possible that altered body composition may be the single most important factor in reducing vascular risk; it has been estimated using the Framingham model that the GH-induced difference in WHR could represent a 3-4 % decrease in the incidence of coronary heart disease over 10 years (188, 191, 192). However, it should be stressed that this is a theoretical projection and it is not clear whether these data apply to GH-deficient patients.

F: GROWTH HORMONE REPLACEMENT THERAPY

Response and Variability to Growth Hormone Replacement therapy

Variable response has been recorded in children with short stature treated with growth hormone. Response varies regardless of the aetiology of GHD: idiopathic, Turner's syndrome, intrauterine growth retardation, or small for gestational age infants.

The effect of GH replacement on body composition has been extensively studied (171, 193) and beneficial effects have been almost universally observed. Adults with GHD demonstrate highly heterogeneous biochemical and clinical responses to treatment with rhGH. Wallymahmed *et al* (126) reported improvement in self-esteem scores following GH replacement therapy along with significant improvements in body composition in GHD patients while Deepak *et al* reported significant improvement in peripheral inflammatory and cardiovascular risk markers in the same North West England patient population (194).

Some patients seem to have little improvement in quality of life and body composition from GH therapy despite increases in the serum IGF-1 into the normal range, while other patients with untreated severe GHD are asymptomatic.

It is possible that these different clinical responses to GH in adults with GHD reflect a spectrum of GH sensitivity.

Effect of GH replacement on Quality of Life

The psychological well-being and QoL of GH-deficient patients and the effects of GH replacement have received considerable attention in recent years.

Quality of life is usually accessed via self-administered questionnaires that reflect a variety of health-related, economic, and social factors. Quality of life measures may be broadly correlated with, but are different from, assessments of affect or cognition. Quality of life evaluations of GHD patients have shown a high degree of variability. For example, in the untreated state, some patients reported severe impairment in quality of life while others reported their quality of life as normal (195). In particular, significant impairment in quality of life was less frequently observed in adults with childhood-onset GHD (162). The area of quality of life most likely to be affected by GHD was energy and vitality (163) . Disease-specific quality of life assessment questionnaires have been validated and are now widely used (159, 196). In most studies, self-perceived well-being and QoL have been assessed using these validated questionnaires, and comparisons have been made with controls of matched age, sex, and socio-economic status (197).

Decreased psychological well-being has been reported in hypopituitary adults who have had replacement of all hormone deficiencies with the exception of GH (198). Comparison was made between the psychological well-being in 86 adults with GH deficiency and 86 age-matched controls using the Nottingham Health Profile (NHP) (199); the GH-deficient patients reported less energy, greater emotional lability, more difficulties with sexual relationships, and a greater sense of social isolation than the control subjects. Stabler *et al.*(198) evaluated psychosocial adjustment in adults with GH deficiency and in age-, sex-, socio-economic and height-matched controls. Those with multiple pituitary hormone deficiencies showed less openness and less assertiveness compared with controls.

A double blind, placebo-controlled study by McGauley et al was the first to demonstrate that rhGH (0.5 IU/kg/week; 12.5 μg/kg/day) was associated with an improvement in mood and energy levels in GHD adults (163). In the study, all three instruments used, the NHP (to assess general health and well-being), the Psychological and General Well-Being Schedule (PGWB), and the General Health Questionnaire, showed significant improvements in subjective well-being and QoL after 6 months of rhGH. The study was performed without knowledge or expectation of the psychological effects of GH therapy, issues that may be relevant in the interpretation of subsequent studies.

Whitehead *et al.*(200) examined the effects of rhGH (0.25 IU/kg/week; 12.5 μg/kg/day) on well-being of 14 adults with GH deficiency in a 6-month, double blind, placebo-controlled, cross-over trial using the NHP and the PGWB questionnaires. No significant changes in psychological well-being were observed, but many patients failed to demonstrate a significant increase in IGF-1, indicating that noncompliance with GH may have accounted for these findings. Two QoL-rating scales [Comprehensive Psychological Rating Scale (CPRS) and Symptom Checklist-90], examined the effects of 6-month GH replacement (0.25 IU/kg/week; 12.5 μg/kg/day) in 26 adults with GH deficiency in a double-blind, placebo-controlled, cross-over study (201). GH treatment was associated with a significant improvement in the CPRS, but not in the Symptom Checklist-90. The CPRS and General Health Questionnaire-90, were used to examine the effects of GH replacement (0.18–0.35 IU/kg·/eek; 9–17.5 μg/kg/day) on 40 adults in a randomized controlled trial (202). The initial 6 months were randomized, but thereafter, patients continuing on GH replacement were followed for 18 months. Those initially randomized to placebo reported greater morbidity at baseline, and placebo was associated with a significant improvement in CPRS compared with GH after 6 months of therapy. Compared to the baseline score, patients

receiving GH reported an improved CPRS score after 6 and 12, but not 18, months of therapy.

Burman *et al.* (156) examined psychological capacity and sense of well-being using the Hopkins Symptoms Check List (HSCL), the NHP, and the PGWB in 36 adults with GH deficiency and 36 matched controls. Those with GH deficiency reported lower QoL, as assessed by the HSCL and NHP questionnaires. The severity of their distress correlated positively with the duration of GH deficiency. Twenty-one of the patients entered a cross-over, double blind, placebo-controlled study of the effects of GH replacement (0.25 IU/kg/week; 12.5 μg/kg/day) on these measures. The HSCL score improved during the placebo phase, but fell further during active treatment. Active treatment was associated with improvement in the energy and emotional subsections of the NHP. In addition, spouses of the patients reported improved mood and behavior in the subjects during the GH treatment phase. An uncontrolled study with a small sample size suggested that GH replacement may result in improved memory in adults with GH deficiency (203).

The direct mechanisms behind alterations in perceived QoL remain unknown. Recently, treatment of GHD adults has been shown to alter levels of vasoactive intestinal polypeptide and the dopamine metabolite, homovanillic acid, as well as elevate ß-endorphin levels in the cerebrospinal fluid, but whether these changes are responsible for improvements in mood and well-being is not yet known (204). GH, IGF-1, and the IGF-binding proteins may have direct effects on the nervous system. In addition, abnormal sleep patterns have been described in GH-deficient adults, with restoration to normal patterns after GH replacement (205, 206).

In summary, some studies showed definite benefit after patients received GH replacement therapy, but in others improvements were more limited or no improvement was seen (207),

(208), (196), (195), (163), (209). The degree of improvement in quality of life is generally proportional to the deviation from normality at the outset (210, 211) but shows no correlation with the degree of improvement in IGF-1 levels (196). In practice, this means that if the patient's quality of life is normal at baseline, no improvement will be seen with GH replacement (209). This is shown in the NICE guidelines for the management of AGHD in which rhGH is only recommended for patients who have demonstrated an impaired score of $\geq$11/25 on the QoL-AGHDA questionnaire(212). It has also been shown that much of the improvement in quality of life occurs within the first 3 months of GH replacement (211, 213). However, some long-term studies have shown sustained benefit in some aspects of quality of life among treated patients as compared with untreated patients (214).

SECTION 4: THE GROWTH HORMONE RECEPTOR AND THE GROWTH HORMONE RECEPTOR GENE

The growth hormone receptor gene (GHR) is the principal regulator of growth hormone (GH) sensitivity in health. It is a single membrane-spanning cell surface protein member of the class 1 cytokine receptor super-family. The human GHR gene consists of 9 coding exons (numbered 2-10) spanning at least 87kb of chromosome 5. Exon 2 encodes the signal peptide; exons 3-7 encode the extracellular domain made up of 246 amino acids; exon 8 encodes the single transmembrane domain; and exon 9 and part of exon 10, the intracellular or cytoplasmic domain.

There are 2 isoforms of GHR in humans, generated by retention or exclusion of exon 3 during splicing: a full-length isoform and an isoform that lacks exon 3 (GHR-d3). The generation of 2 transcripts that differ by the skipping of a coding exon results from homologous recombination, which mimics alternative splicing between the 2 retroviral sequences that flank the skipped exon(215). The allele encoding GHRd3 is specific to

humans. Results of the studies of Pantel et al, 2003(216) supported the hypothesis that the GHRd3 isoform is transcribed from a GHR allele carrying a genomic deletion of exon 3 rather than by alternative splicing.

Mutations at the level of the GH receptor

Subtle mutations in the growth hormone 1 (GH1) gene have been regarded as a comparatively rare cause of short stature. Mutations in the GHR gene have been demonstrated as the cause of Laron syndrome, also known as the growth hormone insensitivity syndrome (GHIS)(217). A number of point mutations have been identified in this gene: missense, nonsense and abnormal splicing. Most of the mutations are in the extracellular domain of the GHR.

A large number of mutations have already been identified in the GHR gene of patients with growth hormone insensitivity. None of these defects which are spread over the entire GHR gene sequence involves exon 3. The functional importance of the GHR domain encoded by exon 3 remains unknown(218).

A: POLYMORPHISM OF EXON 3 OF THE GHR GENE

The GHR is an obvious candidate gene to influence the response to rhGH. GH is a major determinant of postnatal growth and GHR mediates the majority of GH actions (219).

The GHR consists of an extracellular domain of 246 amino acids, a single transmembrane domain and a cytoplasmic domain. The encoding gene has 9 exons, but there are 2 isoforms of the GHR in humans generated either by deletion of exon 3 of the gene resulting in 3 genotypes (220):

- full-length isoform only with retention of exon 3 on both alleles, i.e. homozygous GHR*fl* (full-length GHR – GHR*fl*; NM_000163);

- short isoform only with exclusion of exon 3 on both alleles, i.e. homozygous *GHRd3* (exon 3 deleted GHR – GHR*d3*; AF210633); and
- an approximately 1:1 combination of the 2 isoforms, i.e. heterozygous GHR-*d3/fl*.

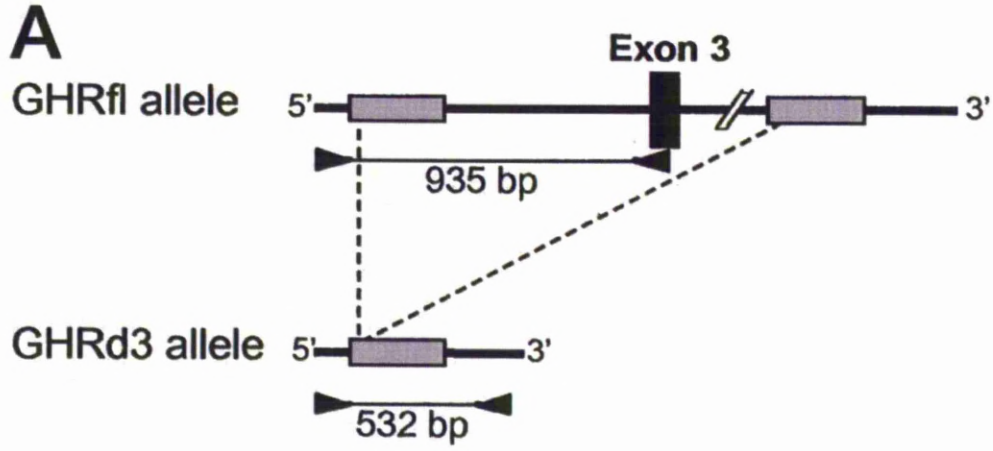


Figure 1.6: Isoforms of exon 3 of GHR gene
[Source: Adapted from Pantel et al. J Clin Endocrinol Metab 2003 88(4): 1705-1710.] (216)

Population studies have shown that the heterozygote frequency is between 25-40%, while the GHR-d3/d3 genotype occurs in 7-15% of the population (3, 216, 221, 222). The two isoforms of GHR, are expressed in the placenta and appeared to be due to alternative splicing. The importance of the region coded for by exon 3 is unknown. The region is conserved in GHR proteins in mammalian species but is absent in prolactin receptor. This pattern of evolutionary conservation suggests that the loss or retention of exon 3 could affect receptor expression or function, specifically by affecting binding of human growth hormone, receptor processing, transport, stability, binding to other ligands, dimerization of GHR monomers or signal transduction. After the dimerization of the two transmembrane chains to form functional GHR, homozygous individuals have either GHR-*fl* or GHR-*d3* homodimers on their cell surfaces, whereas heterozygotes have GHR-*fl* homodimers, GHR-*d3* homodimers and GHR-*d3/fl* heterodimers. Its prominent function in growth hormone

signalling and the frequency of its polymorphic variation makes GHR a good candidate for gene involvement in the pharmacogenomics of growth hormone therapy.

Stallings-Mann et al (2) found no change in the expression of the short form when placentas from different stages of gestation were examined, suggesting that splicing was not developmentally regulated. However, when isoform expression patterns were examined in each component of a given placenta, it was evident that alternative splicing of exon 3 is individual-specific. Surprisingly, this appears to be the result of a polymorphism in the GHR gene. Analysis of the expression of the full-length and short forms found results consistent with simple Mendelian inheritance of 2 differing alleles in which exon 3 is spliced in an 'all or none' fashion(2). It was concluded that the alternative splicing of exon 3 in GHR transcripts is the result of an unusual polymorphism that significantly alters splicing of the transcript, and that the relatively high frequency (approximately 10%) of homozygosity for the allele producing transcripts lacking exon 3 suggests the possibility that it may play a role in polygenically determined events, i.e., may have a selective advantage under some circumstances. A genetic polymorphism resulting in deletion of an entire exon from an mRNA without compromising structure or function of the resultant protein is unusual. Stallings-Mann et al (2) noted that exon 3 encodes a segment of the extracellular domain that is 22 amino acids long, and its removal results in the substitution of an aspartic acid for the alanine residue at the junction of exons 2 and 4. Exon 3 is not highly conserved among GHRs, and a homolog does not exist in the closely related prolactin receptor (OMIM 176761). Placentas showing homozygosity for the deletion were obtained from women who gave birth to apparently normal children.

The relevenace of exon 3 polymorphism in clinical practice

After Pantel et al (220), identified polymorphism of exon 3 of the GHR gene in 2000, there have been several studies to try to determine its clinical significance. Those studies have tried to determine the effect of exon 3 polymorphism of the GHR on response to growth hormone therapy, in children with short stature and those with growth hormone deficiency.

A number of studies in children have shown that this polymorphism may influence GH sensitivity during rhGH therapy for GH deficiency (GHD), Turner's syndrome, idiopathic short stature and those born small-for-gestational age. The subjects with the *d3/d3* or *d3/fl* genotype demonstrated a superior growth response during rhGH therapy compared to those with the *fl/fl* genotype (3, 221, 223, 224). However, there are also a number of studies in similar patient populations where this association was not observed, and thus the true effect of this polymorphism on GH sensitivity in rhGH treated children remains uncertain (222, 225). It has been demonstrated in multiple studies that this polymorphism does not influence final adult height in healthy subjects (226, 227).

Adults with GHD demonstrate highly heterogeneous biochemical and clinical responses to GH replacement therapy.

Some patients seem to have little improvement in quality of life and body composition from GH therapy despite increases in the serum IGF-1 into the normal range, while other patients with untreated severe GHD are asymptomatic.

It is possible that these different clinical responses to GH in adults with GHD reflect a spectrum of GH sensitivity which may be related to the polymorphism of the GHR gene.

There have also been various studies to determine the significance of this polymorphism in other clinical scenarios other than GHD. Schmid *et al* determined that d3-GHR acromegalic patients had lower GH levels (228). Bernabeu's study of 44 acromegalic patients determined that the deletion of exon 3 led to better response to pegvisomant

therapy(229) while recent review of 127 patient by Filopanti *et al* showed no association between d3GHR and response to PEG-V therapy(230). Wassenaar *et al* on the otherhand determined that in patients with long-term cured acromegaly, the d3-GHR polymorphism was associated with an increased prevalence of irreversible comorbidities of acromegaly(231).

Studies reviwing the effect of the polymorphism on cardiovascular markers have shown association with lower BMI and better glucose tolerance with d3-GHR(232). In a review of subjects with normal (NGT), impaired glucose tolerance (IGT) and type 2 diabetes, Strawbridge et al(233), demonstrated that the d3/d3 allele appeared to be preventive of T2DM. However, when other factors caused overt T2DM, the d3-GHR allele confers a phenotype indicative of metabolic disorder. This study supports the hypothesis that the two GHR alleles by their inclusion or exclusion of exon 3 are functionally different.

There associciation of this polymorphism to other clinical conditions such as polycystic overian syndrome(234), coronary artery disease(235) and breast cancer(236) have all been studied with varying results. Overall results from these studies have been inconclusive with no clear and consistent association between exon 3 polymorphism of the growth hormoe receptor gene and the above mentioned disease states.

To date, the functional importance of this common polymorphism is remains undetermined and with the polymprphism occurring late in primate evolution(216), the significance may be yet to be discovered.

In this research study, we set out to investigate the frequency of this polymorphism and to determine its influence of response to rhGH in the local AGHD population using both quality of life and biochemical parameters.

CHAPTER 2

SUBJECTS, MATERIALS AND METHODS

Subjects

GHD Patients

Patients were recruited from the Endocrine Unit of the University Hospital Aintree NHS Foundation Trust and the Joint Neuroendocrine Service of the Walton Centre for Neurology and Neurosurgery (WCNN), both in Liverpool, United Kingdom. The WCNN is a tertiary referral centre for the Mersyside, North Wales and Cheshire regions of the UK. This neuroendocrine service is jointly run by consultant endocrinologists and a consultant neurosurgeon. Patients with various intracranial pathologies initially seen and managed either by surgery and/or radiotherapy by the neurosurgical team are referred to the endocrinologist for assessment and management of their residual endocrine function.

Diagnosis of hypothalamic-pituitary dysfunction would have been made from baseline measurements of the anterior pituitary hormones and dynamic pituitary function testing and if required, was done using glucagon stimulation test for assessment of ACTH and GH axes.

Patients with hypothalamic- pituitary disease were identified to have severe GHD on the basis of a peak GH response of <9mU/l (<3ug/L) following the administration of 1mg subcutaneous glucagon (134, 147). Patients with impaired GH levels on dynamic testing and AGHDA score $\geq$ 11/25 were offered rhGH as per current recommendations.

Consequently, the patients attending the WCNN service who have been diagnosed with GHD fall into 2 main groups:

1. GHD patients already receiving GH therapy
2. GHD patients without symptoms and not requiring therapy

One hundred and seventy-three white Caucasian patients with hypothalamic-pituitary disorders (mean age 50 years, range 16-75 years) were studied. The clinical history for all participants was documented from their medical notes.

Control Population

A control group representative of the normal adult population in the United Kingdom was recruited from the local population in Liverpool. One hundred healthy volunteers were recruited from the University Hospital Aintree staff and relatives. All the volunteers were Caucasian adults. Volunteers were required to read an information leaflet (see appendix), fill a brief form detailing relevant medical history and sign a consent form. All recruited volunteers had no significant medical history and were not taking any medications that could alter GH secretion.

Inclusion Criteria for Patients

- Patients with multiple pituitary hormone deficiencies were eligible for inclusion if other hormone treatments were optimised.
- Patients with GHD aged 16-75years being managed in the Endocrine clinic at the University Hospital Aintree and also at the Joint Neuroendocrine Service at the Walton Centre for Neurology and Neurosurgery (WCNN).
- Healthy adult (aged 16-75years) volunteers were recruited for the control group.

Exclusion criteria for Patients

- Patients with other pathologies or receiving medications likely to impact on general well-being or body composition, including patients with known malignancies, patients receiving glucocorticoid therapy other than for hormone replacement.
- Patients who were unwilling to participate in the study.
- Patients who were unwilling to answer the questionnaires.

Ethics

Ethical approval was sought and obtained from the Local Research Ethics Committee for this study (Ref no: 06/Q1508/25). Written informed consent was obtained from all patients before inclusion in the study. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

Anthropometric measurements

Waist-hip ratio and waist circumference were measured by a single observer. Weight was measured using electronic scales in light clothing without shoes. Height was measured as the distance from the top of the head to the bottom of the feet (no shoes) using a fixed Harpendon stadiometer. BMI was calculated as the weight (kg) divided by the square of the height (m). Waist circumference (cm) was taken with a tape measure as the point midway between the costal margin and iliac crest in the mid-axillary line, with the subject standing and breathing normally. Hip circumference (cm) was measured at the widest point around the greater trochanter. The waist-to-hip ratio was calculated as the waist measurement divided by the hip measurement. Body mass index (BMI) and height standard deviation scores (SDS) were derived from normative data from the British white Caucasian population. The percentage of body fat was measured using a bioimpedance meter (Tanita® Bodyfat Analyzer model TBF-305; Tanita®, Tokyo, Japan).

Figure 2.1: Model of Tanita® Bodyfat Analyser model TBF-dfd305 used in this study
(Source of Image: www.bodyfatscalereviews.org)

Health Related QOL Questionnaires (see appendices)

Health-related Quality of life tools are the standard methods for determining symptoms of GHD in adults. In this study, QoL was measured in the study population using the disease specific QOL-AGHDA and other generic questionnaires previously validated in adults with GHD (158, 167). The order of completion was the same for all patients and the questionnaires were answered in a single session.

Adult Growth Hormone Deficiency Assessment (QoL - AGHDA) questionnaire (159)

The QoL-AGHDA is a uni-dimensional measure of quality of life, using the needs based model. The QoL-AGHDA is a disease-specific measure for use in adults with GHD. It has a maximum score of 25 with lower scores associated with better quality of life. The QoL-AGHDA has a an internal consistency of 0.93 and *test - retest* variability of 0.93(159).

Hospital Anxiety and Depression (HADS) questionnaire (237)

The HADS questionnaire and the Satisfaction Scale is a self-assessment scale which has been developed and found to be a reliable instrument for detecting states of depression and anxiety in the setting of hospital medical outpatient clinics. Higher scores on this scale are associated with worse quality of life.

Life fulfilment Scale and Disease Impact Scale adapted for GHD patients(158)

This scale is based on methods previously described by Krupinski in 1980(238). The value of Krupinski's approach is the opportunity for patients to weigh the numerous aspects of their quality of life and assess the discrepancy between their actual and desired circumstances. The life fulfilment scale has been shown to be reliable and valid. Lower scores are associated with worse QoL. The Disease Impact Scale is to determine the impact of a chronic disease on the daily life of an individual and therefore this was only administered to the GHD patients and not to the control population.

10-cm Visual Analogue Scale (VAS) (239)

The 10cm VAS was used and modified for energy levels with patients giving scores based on how much energy they felt they had. This scale ranges from 0 cm (no energy) to 10 cm (full of energy). Low scores are associated with worse quality of life.

Treatment with recombinant human growth hormone (rhGH)

Hormone replacement in patients with multiple pituitary hormone deficiencies was optimized before assessment for rhGH treatment. If required on the basis of their anterior pituitary hormone profiles and the presence of DI, patients received standard pituitary hormone replacements such as hydrocortisone, thyroxine, sex steroids and desmopressin. Patients treated with rhGH fulfilled the National Institute for Clinical Excellence (NICE) guidelines of a score $\geq$11/25 on the AGHDA questionnaire to qualify for treatment. The asymptomatic patients were defined as having an AGHDA score <11/25, despite biochemical evidence of GHD and were not on rhGH (212).

Patients treated with rhGH were commenced on commercially available rhGH - Humatrope® (Eli Lilly), Norditropin SimplexX® (Novo Nordisk) or Genotropin® (Pfizer).

Patient education and relevant treatment information was provided priror to commencement of therapy by a dedicated endocrine nurse at the Walton Diabetes Centre Patients were educated on how to administer the daily injections and were encouraged to maintain contact with the endocrine nurse over the initial weeks of therapy. The starting dose of rhGH in adults with GHD was 0.3 mg/day by subcutaneous injection. Dose titration was done in the first three months to achieve an IGF-1 level reference range for sex and gender. Maintenance dose was then given for the next 6 months when a re-evaluation of the patients is recommended (212).

Methods

Patients with GHD were recruited consecutively from the neuro-endocrine clinic at University Hospital Aintree. Volunteers from the hospital environment were also recruited. Exon 3 expression of the GHR gene was determined in all participating groups. All patients and volunteers received written information about the study and written informed consent was obtained (see appendices).

Genetic studies were performed under the supervision of Professor M. Pirmohamed at the University of Liverpool. Genomic DNA was obtained from lymphocytes isolated from peripheral blood. A sample of 9ml of blood was collected from the participants into an EDTA bottle and determination of the genotype at the GHR-exon 3 locus of the gene was by PCR based techniques.

DNA was made available to undertake in the future more extensive analysis of other single nucleotide polymorphisms (SNPs) and haplotypes in the GHR gene, and relate these to the clinical parameters. Future studies looking at downstream genes regulating the actions of the GHR may also be possible in the future.

DNA EXTRACTION

Reagents used for DNA Extraction and Amplification

Triton Lysis Mix

Buffer A: 1M Sucrose (MW = 342.3 g/mol)

To make one litre of 1M sucrose, 342.3g of sucrose was dissolved in one litre of distilled water (dH_2O). The solution was heated in a hot water bath to dissolve all sucrose and then autoclaved.

Buffer B: 200mls 1M Tris (pH 7.5) + 100mls 1M $MgCl_2$ + **200mls Triton X**

To make 1L of 1M Tris, 121.14g of Tris was dissolved in 1 litre of dH_2O giving a molecular weight (MW) of 121.14g/mol.

To make 1L of 1M $MgCl_2$, 203.31g of $MgCl_2$ in 1litre of dH_2O giving a MW of 203.31 g/mol. The mixture was then autoclaved.

To make the Triton Lysis Mix, 25 mls of Buffer B was added to 1litre of Buffer A (1:40 dilution). The Triton Lysis Mix was made fresh for use each time.

Nuclear Lysis Buffer

To make 1 litre of nuclear lysis buffer, 43.8g of 0.75M sodium chloride (NaCl) (MW = 58.4 g/mol) was added to 9.3g of 0.0025M EDTA (MW = 372.2 g/mol) and dissolved in 1 litre of dH_2O the solution was then autoclaved and stored at 4°C.

10% Sodium Dodecyl Sulphate (SDS) solution

Twenty grams (20g) of SDS was weighed in a fume hood and dissolved in 200ml of dH_2O. This was not autoclaved. It was stored at room temperature.

Proteinase K

Proteinase K was purchased from Sigma (P2308 – 100mg) and was packed as 100mg powder. To make 10mg/ml solution, 10ml sterile (autoclaved) dH_2O was added to the powder. Aliquots of 1ml solution were labelled into *eppendorfs* (minifuge tubes) and stored at -20°C.

6M NaCl Solution

NaCl with molecular weight of 58.4 g/mol was used. To make 1 litre, 350.4g of NaCl was dissolved in 1litre of dH_2O and then autoclaved.

6M NaCl is a very saturated solution with crystal sediments. It does not dissolve even after heating in hot water bath.

Tris EDTA (TE) Buffer

TE was purchased from Sigma (T9285 – 100ml 100**X** solution). TE powder contains1.0M Tris-HCL and 0.1M EDTA and has a pH of approximately 8.0. To make up at 1**X** concentration of TE, 0.2ml (i.e. 200µl) of 100**X** Tris EDTA was dissolved in 20ml of sterile dH_2O.

ReddyMix™ PCR Master Mix (1.5mM $MgCl_2$)

PCR ReddyMix™ Master Mix is a ready-to-use master mix for amplifying DNA which removes the risk of contamination and pipetting errors. Each vial contains 1.8ml of 1.1x working concentration PCR Master Mix sufficient for 40 x 50µl reactions. The addition of the template and primers results in a final reaction volume of 50µl, containing

1.25 units	Thermoprime Plus DNA Polymerase
75mM	Tris-HCl (pH 8.8 at 25°C)

20mM	$(NH_4)_2SO_4$
1.5mM	$MgCl_2$
0.01% (v/v)	Tween® 20
0.2mM	each of dATP, dCTP, dGTP and dTTP

Precipitate and red dye for electrophoresis

Primers for DNA Amplification

DNA amplification was performed with a multiplex PCR procedure using the following primers: forward primer G1 (5'-TGTGCTGGTCTGTTGGTCTG-3'), [nucleotides 3285-3304] and reverse primers G2 (5'-AGTCGTTCCTGGGACAGAGA-3'), [nucleotides 6534-6515] and G3 (5'-CCTGGATTAACACTTTGCAGACTC-3'), [nucleotides 4219-4196]. The G1, G2 and G3 primers are described in GenBank accession no. AF155912. DNA was amplified using a multiplex strategy described by Pantel et al(215). Primers were purchased at Invitrogen® and Certificate of analysis is shown in *Appendix 7*. The primers were diluted to 5µmol concentration and stored at -20oC.

HyperLadder® IV from Bioline

HyperLadder IV allows determination of molecular weight and DNA quantification and is specially designed for short fragments such as PCR products. HyperLadder IV produces a pattern of 9 regularly spaced bands, ranging from 100 to 1,000bp. The 1,000 band has a higher intensity than the others to allow easy identification and each band is an exact multiple of 100bp.

Procedure for DNA extraction:

DNA was extracted from whole blood which had been stored in EDTA bottles at -20°C. The blood was thawed for 30-45 minutes before use. One ml of blood was pipetted into a cryovial as reserve before starting any DNA extraction. In cases when the blood was partially clotted, it was first homogenised (by vortexing). The thawed blood was poured into a labelled plastic 50 ml falcon centrifuge tube and filled with ice cold sterile distilled H_2O (i.e. autoclaved dH_2O). This was then placed on ice for 30 minutes and afterwards centrifuged at 6000 rpm for 15 minutes at 4°C. Care was taken to note that a pellet had been obtained first and then the supernatant was discarded. The pellet was re-suspended in 25 mls of ice cold Triton lysis mix pH 7.7 using a pastette. This was again placed on ice for 30 minutes and then centrifuged at 6000 rpm for 10 minutes at 4°C and the supernatant discarded. What was left was a clean, white pellet of nuclei. This pellet was re-suspended in 9 mls of pH 8.0 Nuclear Lysis buffer using a pastette. 500 µl of SDS (10%) and 200 µl proteinase K (10 mg/ml) were added. The powerful proteolytic activity of proteinase K combined with the denaturing ability of SDS lysed the white cell membranes and the nuclear membranes, thus releasing the DNA from the nucleus.

This was then incubated at 55°C for 3 hrs. Thereafter, if the preparation looked clean, (colourless, or yellow ringed) 1/3 volume of 6M NaCl was added and shook vigorously for 20 seconds. This was then centrifuged at 6000 rpm at 4°C for 15 minutes. If it looked dirty, 10 mls of Phenol: Choloroform 1:1 was added and the mixture spun for 10 mins at 6000 rpm. The supernatant was removed and 1/3 vol. of 6M NaCl was added to it. This mixture was shaken vigorously for 20 seconds and then spun at 6000rpm at 4°C for 15 minutes.

The supernatant was carefully poured (or pipetted) into a clean labelled falcon centrifuge tube, taking care not to disturb the pellet and about 2 vols. of 100% ethanol was added. The tube was swirled fairly vigorously and the DNA precipitated out as a white clump. The

DNA was carefully hooked out using a syringe with needle or pipette tip and placed in a minifuge tube. One ml of 70% ethanol was then added to wash the DNA, and shaken gently. This was spun at 5000 rpm for 1 minute to pellet the DNA. The ethanol was removed completely using a pipette tip. The DNA was then dried for 10 minutes at 37°C (It was important not to over-dry the DNA since it would not readily dissolve in TE) or left to dry in the safety cabinet at room temperature. 450 µl of sterile TE was added and stored at 4°C for 2 weeks.

Measurement of DNA concentration and dilution

After 2 weeks, the DNA concentration was measured using a spectrophotometer. The UV and Vis were switched on and allowed to warm up for up to 1 hour. The cuvette was rinsed out with distilled water. 100µl of 1x TE was blanked in the metre. Then 2µl of DNA was added to 100µl of 1xTE in the cuvette and mixed well with a pipette tip. The absorbency at 260nm was read and the mean of 3 readings was recorded. The A260/A280 ratio had to be greater than 1.7.

DNA Dilution:

Final stock DNA required was 20mg/µl (µg/ml) and final volume of DNA required was 200µl. The amount of DNA required to make up 200µl solution was calculated.

The DNA solution was then stored at 4°C and the remaining DNA was stored at –20°C for future use.

DNA extraction using Chemagen®

Genomic DNA was extracted from whole blood using magnetic bead technology and Chemagic Magnetic Separation Module I (Chemagen®, Auto Q Biosciences Limited, Berkshire, UK) according to the manufacturer's protocol for 10 ml of blood.

Preparation of blood Lysate

Lypophilised protease was dissolved in water and stored at 4oC which was viable for4-6 weeks. 50µl of protease was added to each tube containing 10ml of whole blood. 8ml of lysis buffer was then added. This mixture was commenced on lysate mixing for Chemagic DNA blood protocol for 20minutes. 28ml of binding buffer solution was then added to the lysate and then 1.2ml of magnetic beads. This mixture was commenced on automated isolation process according to Chemagic protocol. 1ml of DNA solution was then isolated and stored at 4°C.

DNA amplification with polymerase chain reaction (PCR)

1ul of 20ng/ul genomic DNA was added to 24ul reaction mixture (ReddyMix™ Master Mix, ABgene, UK) of 1.5mM MgCl2, 0.5mM each dNTP, 1ul of 0.2mM of each primer. The cycling programme performed on a G-storm Thermal Cycler PCR machine (Licensed is from Applied Biosystems, California, USA) was as follows: denaturation for 2mins at 94°C followed by 35cycles of 94°C for 30sec, 60°C for 30sec and 72°C for 90sec. This was followed by a final extension step of 7mins at 72°C. It was then cooled for 10°C for 30mins.

The presence and size of amplification products was determined by electrophoresis on 1% agarose gel stained with ethidium bromide. 1% agarose was prepared using 0.5% tris borate EDTA (TBE). 0.8g of agarose was dissolved in 80ml of 0.5TBE. The mixture was

dissolved and 8ng (10mg/ml) ethidium bromide. The mixture is poured in an electrophoresis plate with comb trays inserted. When cooled the gel sets. The tank is filled with 0.5TBE then 12µl of amplified DNA solution in inserted in each well with one well containing 5µl of HyperLadder® IV from Bioline. The electrode is set at 100V for up to 30 minutes.

The fl allele (GHR*fl*) is represented by a 935-bp fragment and the d3 allele (*GHRd3*) by a 532-bp fragment. When a homozygous *d3/d3* genotype was detected (a single band corresponding to 532-bp) and/or when a band potentially corresponding to 935-bp product was mildly amplified, a second PCR using G1 and G3 primers was carried out, which amplified GHR*fl* alleles. Quality control assessment included using both positive and negative controls in each batch of samples (sample of alleles following electrophoresis shown in appendix).

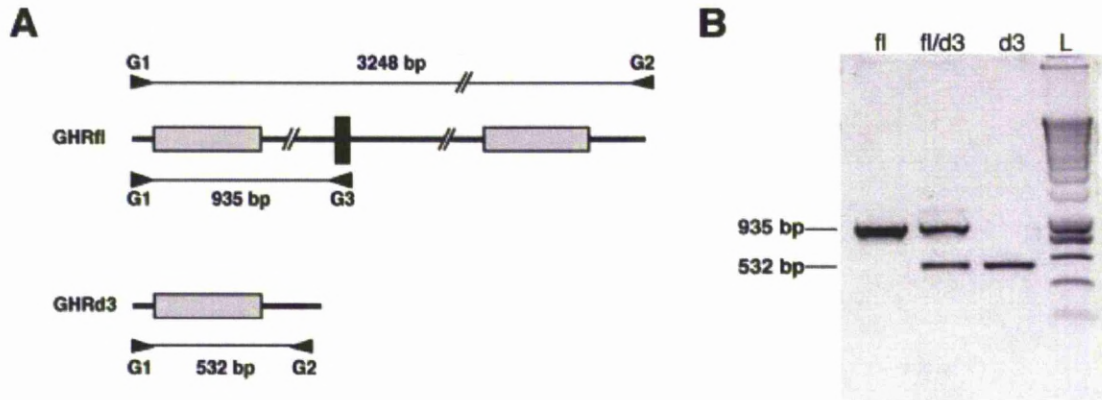


Figure 2.2: Genotype assay at the GHR exon 3 locus
(Source: J. Biol. Chem., Vol. 275, Issue 25, 18664-18669, June 23, 2000) (220)

A schematic representation of the human GHRfl region including exon 3 (*black box*) and the repeated elements (*grey boxes*) is shown in Figure 2.2. The GHRd3 allele contains a single copy of the repeat (*grey box*). The position and orientation of primers G1, G2, and G3 used in the multiplex PCR assay are indicated by *arrowheads*. *B*, under specific experimental conditions (*i.e.* denaturation 94 °C, 30 s; annealing 60 °C, 30 s; and elongation 72 °C, 90 s), primers G1 and G2 allowed the amplification of GHRd3 alleles only, whereas primers G1 and G3 amplify GHRfl alleles. The homozygous GHRfl, heterozygous GHRfl/GHRd3 and homozygous GHRd3 genotypes are denoted by *fl*, *d3/fl*, and *d3*, respectively.

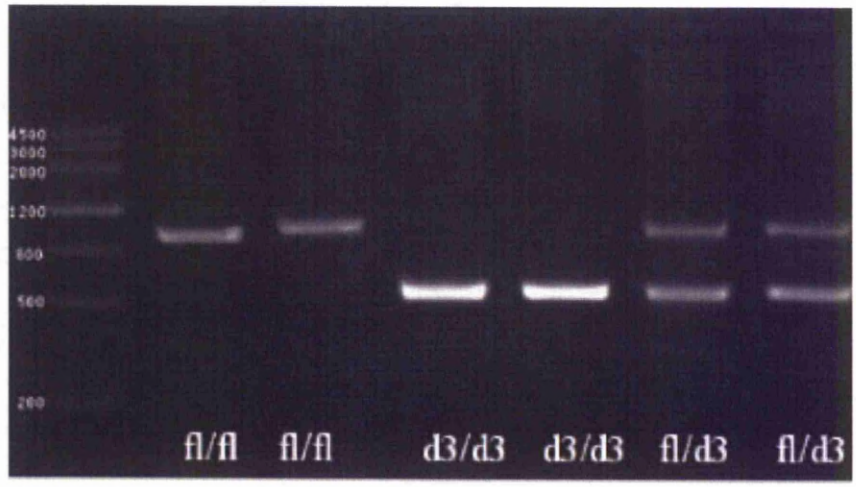


Figure 2.3: Electrophoresis of PCR amplified DNA fragments. The 935-bp band indicates fl allele, the 532-bp band indicates the d3 allele (240)

Hormone measurements

Serum GH levels were measured following the administration of 1mg subcutaneous glucagon (glucagon stimulation test) in all GHD patients prior to commencement of rhGH therapy. Serum GH and IGF-1 levels were analysed in the hospital laboratory using chemiluminescent immunometric assays using IMMULITE 2000 (Siemens Medical Solutions Diagnostics).

Statistical analysis

Results are expressed as percentages and mean ± SD. Continuous data analysis for a difference among the *GHRd3*/GHR*fl* genotypes was calculated using ANOVA (with post-hoc Bonferroni correction for multiple testing). Categorical data analysis including the *GHRd3*/GHR*fl* allelic and genotype frequencies was performed by the chi-square test. Statistical analyses were performed using SPSS15.0 for Windows.

CHAPTER 3

ASYMPTOMATIC GROWTH HORMONE DEFICINECY

Introduction

Two sets of GHD patients are seen in our clinic population; a treated group on rhGH and an asymptomatic group not requiring GH replacement therapy.

In clinical practice, decision on who to commence on rhGH is based on the QoL-AGDHA questionnaire. Patients would have fulfilled National Institute for Clinical Excellence (NICE) guidelines of a score ≥11/25 on the QoL-AGHDA questionnaire to qualify for treatment. Scores of ≥11 are often associated with tiredness, fatigue, mood lability and reduced quality of life. When multiple pituitary hormone deficiencies exist, all other deficient hormones need to be replaced and optimized before patients are assessed for rhGH treatment.

An interesting group of patients however score low on the QoL-AGHDA, that is, <11/25 score despite biochemical evidence of severe GHD (i.e. peak GH response during glucagon stimulation testing < 3ug/L). These asymptomatic patients do not require treatment according to current NICE guidelines.

It is not known why some patients appear not to have impairment in their quality of life despite sometimes very significantly severe growth hormone deficiency. The obvious gene to investigate is the GHR which is the principal regulator of growth hormone (GH) sensitivity. Polymorphism of exon 3 of the GHR has been shown to influence response to human recombinant growth hormone therapy (rhGH) with patients homozygous or heterozygous for exon 3 deletion, *d3/d3 or d3/fl* showing better response to treatment. In patients with asympotomatic GHD, could a higher prevalence of exon 3 deletion improved adaptability to low levels of GH and thus no exhibition of impaired QoL despite biochemical deficiencies?

We hypothesized therefore that a higher prevalence of exon 3 deletion may exist among this population of asymptomatic patients with GHD.

Subjects and Methods

Patients

One hundred and seventy-three white Caucasian patients with hypothalamic-pituitary disorders (mean age 50 years, range 16-75 years) were studied. All patients had severe GHD defined as peak GH <9mU/l (<3ug/L) following the administration of 1mg subcutaneous glucagon (134, 147). The clinical history for all participants was documented from their medical notes.

Health related QOL questionnaires

Quality of life tools are the standard methods for determining symptoms of GHD in adults. QoL was measured in the study population using 4 validated questionnaires: the Adult Growth Hormone Deficiency Assessment (AGHDA) questionnaire(159), the Hospital Anxiety and Depression (HADS) questionnaire, the Life fulfilment Scale and Disease Impact Scale adapted for GHD patients(158). Finally, energy levels were determined using a 10-cm Visual Analogue Scale (VAS). The order of completion was the same for all patients and the questionnaires were answered in a single session. Higher scores are associated with worse quality of life in the AGHDA questionnaire, the HADS and the Satisfaction Scale, and lower scores with worse QoL or energy levels in the Disease Impact Scale and VAS, respectively.

GH replacement therapy

Hormone replacement in patients with multiple pituitary hormone deficiencies was optimized before assessment for rhGH treatment. Treated patients fulfilled the National Institute for Clinical Excellence (NICE) guidelines of a score ≥11/25 on the AGHDA questionnaire to qualify for treatment. The asymptomatic patients were defined as having an AGHDA score <11/25, despite biochemical evidence of severe GHD and were not on rhGH.

Genotyping and hormone measurements

Genomic DNA was extracted from 10mls whole blood using magnetic bead technology and Chemagic Magnetic Separation Module I. DNA was amplified using a multiplex strategy. The G1, G2 and G3 primers are described in GenBank accession no. AF155912. The fl allele (GHR*fl*) is represented by a 935-bp fragment and the d3 allele (*GHRd3*) by a 532-bp fragment. Quality control assessment used both positive and negative controls in each batch of samples. Serum GH and IGF-1 levels were analysed in the hospital laboratory using chemiluminescent immunometric assays.

Statistical analysis

Results are expressed as percentages and mean ± SD. Continuous data analysis for a difference among the *GHRd3*/GHR*fl* genotypes was calculated using ANOVA (with post-hoc Bonferroni correction for multiple testing). Categorical data analysis including the *GHRd3*/GHR*fl* allelic and genotype frequencies was performed by the chi-square test. Statistical analyses were performed using SPSS15.0 for Windows.

Results

Population studies

Of the 173 patients studied, 131 were treated with rhGH for impaired QoL. The remaining 42 patients were asymptomatic and did not require rhGH replacement. The mean age of the entire study population was 51 (±13) years with a mean duration since diagnosis of GHD 13 (±10) years. There was a male preponderance of 57% in the population. Tables 3.1, 3.2 and 3.3 show the primary diagnoses, previous treatments, other medical conditions and hormone replacement in the two patient groups.

Comparison of QoL scores between asymptomatic patients and GHD patients

Table 3.4 shows the comparison of measured variables and QoL scores between the asymptomatic patients and those on rhGH. The asymptomatic patients have significantly better QoL scores despite having significantly lower IGF-1 levels. There was however no significant difference in the WHR, BMI and percentage body fat mass between the two groups.

GHR-genotype frequencies and effect of the GHR *d3/fl* genotypes

Ninety five patients (55%) were homozygous for *fl/fl*, 65 (37%) were heterozygous *d3/fl* and 13 (8%) were homozygous *d3/d3*. Table 3.5 shows the distribution and frequency of the different genotypes in treated and untreated populations. There was no significant difference in distribution between the rhGH treated patients and the asymptomatic patients. Table 3.6 shows the comparison between the genotypes for the whole study population. This comparison includes their peak GH levels at presentation, the rhGH dose required to optimize their serum IGF-1 levels (for patients on treatment), their present QoL scores and body composition. There was no significant difference in any of the parameters between

genotypes both with the *d3* isoforms pooled together and analysed separately except for the depression scale among the asymptomatic patients.

Body composition and QoL were compared between genotype groups for patients treated with rhGH and asymptomatic patients (Table 3.6). The asymptomatic GHD patients with the *d3* isoform had significantly lower depressions scores than those with the *fl/fl* isoform. However, no other differences in QoL scores were found in the *d3* isoform patients compared to the fl/fl patients in the treated and untreated populations.

Discussion

Controversy exists as to the role of the GHRd3 polymorphism on the response of GHD patients to treatment. Some studies in children have demonstrated a superior growth velocity in individuals with the *d3* isoform(3, 224, 241) while other studies report there is no significant difference between these groups(222, 225). For GHD adults in the United Kingdom, apart from biochemical evidence of GHD, the decision to commence and continue treatment are based on the demonstration of impaired QoL from NICE approved validated questionnaires. Two sets of GHD patients are seen in our clinic population; a treated group on rhGH and an asymptomatic group not requiring GH replacement therapy. In this study, we speculated that adult GHD patients with exon *3* deletion are less likely to be symptomatic from GHD than those without. We therefore studied the relationship between QoL, the need for rhGH therapy and deletion of exon *3* in the GHR gene. We were also investigated the relationship between exon 3 deletion and other markers of GHD: serum IGF-1 prior to rhGH therapy and anthropometric measures.

The prevalence of the three GHR *d3* genotypes in this population was comparable to previous studies in which up to half of the population is homozygous for the *fl* allele (221,

242, 243). As previous studies in childhood suggest that patients who delete exon 3 on one or both alleles *(d3/fl and d3/d3)* have a similar growth response to rhGH, data from these patients were analysed together and separately. The results of this study do not support our hypothesis that patients with the d3/fl or d3/d3 genotype are less likely to need rhGH therapy to improve quality of life than those with the fl/fl genotype (shown in Table 3.4). The difference in depression scale between the two genotype groups is probably of no clinical significance because scores of 0 to 7 on the HADS-depression scale are classed as normal with 8 to 10 being borderline normal and 11 to 21 being abnormal(237). Data from this study also suggest that the GHR-d3 polymorphism does not influence stimulated GH levels prior to rhGH therapy or body composition.

This study suggests that in the adult population with GHD factors other than the GHR *d3* polymorphism influence the QoL. There are multiple causes of hypopituitarism in GHD adults and patients receive multiple and prolonged treatments, attending regular and repeated follow-up MRI/CT scanning which, in themselves, generate anxiety. As a result, determinants of QoL in adults are probably multi-factorial and may in fact be difficult to relate to a single cause such as the GHR*d3* polymorphism. Coexisting medical conditions are also likely to impact on QoL. It is also important to observe that while in children, response to treatment is easily measured by growth velocity, QoL measures remain the best way of monitoring response to treatment in adults with GHD. However in many patients, QoL is difficult to measure and interpret, and may lack sensitivity.

The data show that despite adequate hormone replacement, patients on rhGH continue to have suboptimal QoL. This has been reported in previous studies showing that the QoL scores improve and then plateau after a few years on treatment (126, 167). Both

populations of GHD patients have multiple hormone deficiencies and are receiving other hormone replacements.

To date, results relating the GHR*d3* polymorphism and growth hormone response are inconclusive. Further studies are needed to investigate this and other polymorphisms in both children and GHD adults. In conclusion, we have demonstrated that the deletion of exon 3 in the GHR gene does not influence QoL and energy levels in adults with GHD.

Table 3.1: Diagnoses and previous treatments of the 42 patients with asymptomatic severe GHD

Diagnosis	Total	Surgery	Irradiation
Non-functioning Pituitary adenoma	17	16	7
Craniopharyngioma	2	1	1
Prolactinoma	8	4	4
Pituitary apoplexy	5	2	-
Miscellaneous	10	4	6

Miscellaneous: 1 Cushing's disease,1Rathke's cyst, 1 acromegaly, 1 empty sella, 1 arachnoid cyst, 1 chordoma, 1 hypothalamic harmatoma, 1 pineal tumour,1 suprasellar tumour and 1 cerebral seminoma

Table 3.2: Primary diagnosis and previous treatments of the 131 patients treated with rhGH

Diagnosis	Total	Surgery	Irradiation
Non-functioning Pituitary adenoma	45	42	22
Craniopharyngioma	23	19	11
Prolactinoma	11	4	3
Pituitary apoplexy	4	1	0
Rathke's cyst	3	3	0
Astrocytoma	4	2	4
Medulloblastoma	4	4	4
Miscellaneous	37	20	19

Miscellaneous: Cushing's disease, acromegaly, empty sella syndrome, arachnoid cysts, chordoma, idiopathic isolated GHD, glioblastoma, thalamic tumour, meningioma, Langerhan's cell histiocytosis, HSV encephalitis, pineal tumour, septo-optic dysplasia, nasopharyngeal neuroblastoma

Table 3.3: Coexisting medical conditions and hormone deficiencies of the 173 GHD patients

	Asymptomatic GHD n=42	GHD patients on rhGH n=131
Replacement therapy		
Corticosteroids	25 (60%)	91 (69%)
Thyroxine	24 (57%)	79 (60%)
Sex Hormones	22 (52%)	83 (63%)
DDAVP	4 (10%)	28 (21%)
Dopamine Agonists	9 (21%)	12 (9%)
Total hormone replacement	2 (5%)	20 (15%)
Coexisting Medical Conditions		
Diabetes Mellitus	2 (5%)	5 (4%)
Hypertension	12 (29%)	34 (26%)
Dyslipidaemia	24 (57%)	74 (56%)
Epilepsy	2 (5%)	12 (9%)
Previous CVA	1 (2%)	6 (5%)
Ischaemic Heart Disease	2 (5%)	7 (5%)
Asthma	1 (2%)	6 (5%)
Morbid Obesity	1 (2%)	5 (4%)
Osteoarthritis	2 (5%)	4 (3%)
Anxiety/depression	-	3 (2%)
Severe COPD	-	2 (2%)
Peripheral Vascular Disease	1 (2%)	1 (1%)

Table 3.4: Comparison of measured parameters in treated and asymptomatic patients

	Asymptomatic GHD n=42	GHD on treatment n=131	p value
Gender (m:f)	27:15	71:60	
Age (years)	56(16)	50(12)	p=0.01
Mean duration of GHD (years)	11(7)	13(10)	NS
Peak GH (mU/l)	1.9(1.9)	2.6(2.9)	NS
Serum IGF-1 at diagnosis of GHD (nmol/L)	11.1(4.8)	12.4(4.7)	NS
Current serum IGF-1 (nmol/L)	12.0(5.1)	25.5(11.6)	p<0.001
QoL			
AGHDA	5(5)	10(7)	p<0.001
HADS-depression	4(4)	7(5)	p<0.001
HADS- anxiety	4(2)	7(4)	p=0.001
LFSS	43(4)	42(4)	NS
Satisfaction scale	21(5)	25(5)	p<0.001
VAS energy scale (cm)	7.4(2.1)	5.7(2.1)	p<0.001
Measurements			
Height (SDS)	-0.50(1.4)	-0.40(1.3)	NS
BMI(SDS)	2.2(1.1)	2.1(1.0)	NS
WHR	0.97(0.1)	0.93(0.1)	NS
Body Fat (%)	31.6(10.4)	34.8(9.5)	NS

Significance $p<0.05$. NS - not significant, Data are presented as mean (SD)
HADS - hospital anxiety and depression scale, LFSS- life fulfilment and satisfaction scale, VAS-visual analogue scale, BMI- body mass index, WHR- waist-hip ratio.

Table 3.5: Frequency of genotypes in the study population

Genotype	Asymptomatic GHD n=42	GHD on treatment n=131
fl/fl	55%	55%
d3/fl	33%	39%
d3/d3	12%	6%

Table 3.6: Comparison of QoL scores and measured variables between genotype groups in treated and asymptomatic patients (n=173)

Measured variables		GHR genotype fl/fl	d3/fl or d3/d3	p value
Frequency				
	On rhGH	72	59	Ns
	Asymptomatic	23	19	Ns
Age (years)				
	On rhGH	49 (12)	51 (12)	Ns
	Asymptomatic	55 (17)	56 (13)	Ns
Peak GH @diagnosis (mU/l)				
	On rhGH	2.7 (3.4)	2.4 (2.2)	Ns
	Asymptomatic	1.7 (2.0)	2.0 (1.8)	Ns
rhGH Dose (mg)	**On rhGH**	0.52 (0.6)	0.36 (0.2)	Ns
Serum IGF-1 @diagnosis (nmol/l)				
	On rhGH	13.3 (5)	11.1 (4.1)	Ns
	Asymptomatic	10.3 (4)	11.4 (5.1)	Ns
Serum IGF-1 (nmol/l)				
	On rhGH	25.5 (12)	25.5 (11.5)	Ns
	Asymptomatic	11.4 (5)	12.7 (5.3)	Ns
QoL				
AGHDA				
	On rhGH	9 (6)	10 (7)	Ns
	Asymptomatic	5 (4)	5 (5)	Ns
HADS-depression				
	On rhGH	6 (5)	8 (5)	Ns
	Asymptomatic	5 (4)	2 (2)	0.02
HADS- anxiety				
	On rhGH	6 (4)	7 (5)	Ns
	Asymptomatic	3 (2)	4 (3)	Ns
LFSS				
	On rhGH	42 (4)	41 (4)	Ns
	Asymptomatic	43 (4)	43 (4)	Ns

Measured variables	GHR genotype fl/fl	d3/fl or d3/d3	p value
Satisfaction scale			
On rhGH	24 (5)	25 (5)	Ns
Asymptomatic	21 (5)	20 (4)	Ns
Disease Impact scale			
On rhGH	26 (7)	24 (7)	Ns
Asymptomatic	29 (7)	31 (9)	Ns
VAS energy scale (cm)			
On rhGH	5.9 (2.0)	5.3 (2.2)	Ns
Asymptomatic	7.3 (2.0)	7.6 (2.2)	Ns
Measurements			
Height (SDS)			
On rhGH	-0.51 (1.4)	-0.24 (1.2)	Ns
Asymptomatic	-0.31 (1.5)	-0.73 (1.3)	Ns
BMI (SDS)			
On rhGH	2.1 (1.1)	2.2 (1.0)	Ns
Asymptomatic	2.2 (1.2)	2.3 (0.9)	Ns
WHR			
On rhGH	0.92 (0.1)	0.93 (0.1)	Ns
Asymptomatic	0.98 (0.1)	0.95 (0.1)	Ns
% Body Fat			
On rhGH	34.2 (8.8)	35.7 (10.2)	Ns
Asymptomatic	30.9 (11.4)	32.7 (9.4)	Ns

Significance $p<0.05$, NS - not significant, Data are presented as mean (SD)

CHAPTER 4

EFFECT OF GHR GENE POLYMORPHISM ON ADULTS ON GROWTH HORMONE TREATMENT

Introduction

A number of studies in children have shown that the GHR polymorphism may influence GH sensitivity during recombinant human GH (rhGH) therapy for GH deficiency (GHD), Turner's syndrome, idiopathic short stature and those born small-for-gestational age. Thus, subjects with the *d3/d3* or *d3/fl* genotype demonstrated a superior growth response during rhGH therapy compared to those with the *fl/fl* genotype (3, 221, 223, 224). However, there are also a number of studies in similar patient populations where this association was not observed, and thus the true effect of this polymorphism on GH sensitivity in rhGH treated children remains uncertain. It can perhaps be stated with more certainty that this polymorphism does not influence final adult height in healthy subjects (222, 225, 244).

Growth hormone deficiency developing in adult life results in impaired quality of life (QoL) and altered body composition (BC). As in children, it is now standard practice to treat adult growth hormone deficiency with rhGH. In this study, we investigated GHD adults who have been treated with rhGH for more than one year, in order to determine the relationship between genomic deletion of exon 3 in the GHR gene and QoL, body composition and serum IGF-1 levels, and to compare these variables to a healthy adult control population.

Materials and Methods

Patients

One hundred and thirty-one patients with GHD attending the endocrine clinic and receiving rhGH treatment for more than one year were studied. All the patients studied were white Caucasians. All patients had severe GHD defined as peak GH < 9mU/l (3ug/L)(212) after 1mg subcutaneous glucagon(134, 147). The clinical history was documented from medical records.

Controls

A control group of 100 healthy white Caucasian adult volunteers from the local population were recruited for the study. The volunteers had no significant past medical history, family history of pathologic short stature and no previous or existing pituitary disease. They were assessed using the same QoL instruments as the GHD patients and their body composition and GHR genotypes were also determined.

Health related QOL questionnaires

QoL was assessed in both the patient and control populations using 4 validated questionnaires: The National Institute for Clinical Excellence (NICE) approved questionnaire for treatment of GHD, the Adult Growth Hormone Deficiency Assessment (AGHDA) questionnaire(159), the Hospital Anxiety and Depression (HADS) questionnaire, the Life fulfilment Scale and the Satisfaction and Disease Impact Scale adapted for GHD patients(158). The volunteers were not required to fill in the Disease Impact scale questionnaire. Finally, a 10-cm Visual Analogue Scale (VAS) was also used to determine energy levels. The VAS scoring ranged from *0cm* (no energy) to *10cm* (full of energy). This tool has been used in previous studies of adult GHD patients(167). Higher

scores are associated with worse quality of life in the AGHDA questionnaire, the HADS and the Satisfaction Scale, and lower scores with worse QoL and energy levels in Disease Impact Scale and VAS, respectively. The order of completion of questionnaires was consistent and all questionnaires were answered in a single session.

GH treatment

Patients with multiple hormone deficiencies were adequately replaced and treatment optimized prior to commencement of rhGH according to existing national treatment guidelines(245). All patients had also fulfilled *NICE* guidelines of a score $\geq$11/25 on the AGHDA questionnaire before commencing treatment(212).

Genotyping and hormone measurements

Genomic DNA was extracted from 10mls whole blood using magnetic bead technology and Chemagic Magnetic Separation Module I. DNA was amplified using a multiplex strategy. The G1, G2 and G3 primers are described in GenBank accession no. AF155912. The fl allele (GHR*fl*) is represented by a 935-bp fragment and the d3 allele (*GHRd3*) by a 532-bp fragment. Quality control assessment used both positive and negative controls in each batch of samples. Serum GH and IGF-1 levels were analysed in the hospital laboratory using chemiluminescent immunometric assays.

Statistical analysis

Results are expressed as percentages and mean ± SD. Differences between the variables evaluated among the *GHRd3*/GHR*fl* genotypes were calculated using ANOVA (Bonferroni). Differences for the *GHRd3*/GHR*fl* genotype frequencies were analyzed by the chi-square test. Data were analyzed using SPSS 15.0 for Windows.

Results

Population studies and GHR-genotype frequencies

One hundred and thirty-one GHD patients who had been treated with rhGH for more than one year were studied, of whom 71 (54%) were male. The age of the patient population was 50 (±12) years and the mean duration since primary diagnosis in the patient population was 12 (±10) years. One hundred and two patients (78%) had structural hypothalamic-pituitary disorders and sixty-three (48%) of the patient population had received hypothalamic irradiation. Table 4.1 shows the aetiology of GHD in the patients studied. Table 4.2 shows the coexisting medical conditions and hormone replacement therapies.

Figure 4.1 shows the distribution and frequency of the different genotypes in patients and controls. Seventy-two patients (55%) were homozygous for the wild-type allele *(fl/fl)*, 51(39%) were heterozygous for the allele coding the d3 isoform *(d3/fl)* and 8 (6%) were homozygous (*d3/d3*). Of the 100 healthy adult controls, 44% were males with a mean age of 45 (±13) years. The frequency of the genotypes was 53% *(fl/fl)*, 40% *(d3/fl)* and 7% *(d3/d3)*. There was no significant difference in the frequencies of these alleles between the patient and control populations.

Comparison of study population between GHR genotypes

The study populations (patients and controls) were divided into 2 genotype groups, with the *d3* homozygous and heterozygous isoforms grouped together. Comparison was made between the clinical and laboratory parameters and QoL scores between the 2 groups for both patients and controls. There was no significant difference in the measured variables based on the genotype in the patient population except for the LFSS scale (Table 4.3). Similarly in the control population there was no difference in QoL scores and clinical parameters between the different genotypes (not shown). Further analysis of both

population genotypes grouped separately *(i.e. fl/fl, d3/fl and d3/d3)* still showed no significant difference in measured variable.

Further analysis of the volunteer population was carried out to determine the frequency of the genotypes in the extremes of height (i.e. SDS<2.0 and >2.0); no significant difference in the extremes of height was identified.

Comparison of QoL scores between patient and control population

Comparison of measured variables and QoL scores was undertaken between the treated GHD patients and the healthy adult controls (shown in Table 4.4). There were significantly better QoL scores in the control population as compared to the patient population despite being on replacement therapy. Measurements of BMI and height were expressed in mean (SD) standard deviation scores (SDS) for normal adult Caucasian population. Both populations were of similar height, but the GHD patients had a significantly higher BMI and WHR than the control population.

Discussion

Despite several recent publications in the paediatric population, there has been very little interest in the role of polymorphisms in the GHR gene and response to GH therapy in the adult population. Studies investigating the exon 3 polymorphism in the adult population(4, 246) did not address QoL which remains the main indication for the use of rhGH in the United Kingdom. This study aimed to determine the prevalence of the polymorphism of exon 3 of the GHR in the local population and also to analyze whether a relationship exists between genotypes GHR-*d3/d3*, GHR-*d3/fl*, and GHR-*fl/fl* and QoL, body composition and serum IGF-1 levels in GHD adults on stable GH replacement.

A total of 100 healthy Caucasian adults were studied and the prevalence of the GHR-d3 polymorphisms was comparable to previous studies showing up to half of the population

being homozygous for the *fl* allele. Similar frequencies were also seen in the patient population. Previous studies have shown that growth response in GHD children is greater in children who exclude exon 3 on one or both alleles compared to those who have the full length isoform on both alleles(224). Therefore, for this study, analysis was done with the genotypes GHR-*d3/fl* and *d3/d3* grouped together and also separately (data not shown). We acknowledge that the low prevalence of the homozygous d3 *(d3/d3)* precludes proper study of this group.

Despite adequate replacement, patients on rhGH continue to have suboptimal QoL. This has been reported in previous studies showing that the QoL scores improve but do not normalize and then plateau out after a few years on treatment(126, 167). This is not surprising as many of these GHD patients have had surgery and radiotherapy for intracranial pathologies and also have multiple hormone deficiencies and are receiving other hormone replacements. Also, GHD patients have a significantly higher BMI and fat mass than the control population, probably in part due to the metabolic consequences of hypothalamic-pituitary disoders(247, 248). Interestingly however, these features also do not always revert to normal with hormonal replacement. As expected, there was no significant difference in the heights of the volunteers or the patients because GHD was mainly of adult onset.

Comparison was made between the different genotypes [*fl/fl* vs *d3/fl* and *d3/d3*] for all the measured variables in both patients with GHD and in the control population. In the patients, this comparison included peak GH levels at diagnosis following glucagon stimulation tests, the rhGH dose required to optimize their serum IGF-1 levels, their present QoL scores and body composition. There was no significant difference in any of these parameters, except for the LFSS between genotypes both with *d3* isoform genotypes analysed together and separately. The LFSS is a scale that provides the individual an

opportunity to identify the most important areas of their lives and then 'Satisfaction with' and 'Impact on' these areas are then assessed in the subsequent Satisfaction and Impact scales.

These data suggest that the polymorphism in exon 3 of the GHR does not influence peak GH levels during dynamic pituitary stimulation tests prior to commencement of treatment. The polymorphism also does not influence patient response to treatment as determined by their QoL scores and body composition. Finally, this polymorphism did not influence the extremes of height in the control population in agreement with a previous studies(249).

Other polymorphisms such as 504A>G at codon 168 of exon 6 and polymorphism 1576 A>C at codon 526 are common but do not influence response to rhGH treatment in children (225). However, the C.1319G>T polymorphism was found to be associated with therapeutic efficacy in GHD children (250). Additionally, other genes within the GH pathway may also be important, and this relative importance may differ between adults and children. Therefore, further studies to investigate genes within and outwith the GH pathway are needed. This is important as rhGH is expensive; some patients develop adverse effects from therapy, while others show little response. Individualisation of therapy in this group of patients therefore is likely to have multiple benefits.

In conclusion, our data show that exclusion of exon 3 in the GHR gene does not influence adult height, QoL or body composition in the normal adult control population. Similarly, in GHD adults treated with rhGH for >1year, rhGH dose, QoL and body composition, as well as biochemical parameters, were not influenced by GH isoform expressed by the patient. Further studies in adult patients with GHD are required to determine the reasons for variability in response to rhGH.

Table 4.1: Underlying diagnosis and previous treatment of patients with GHD

Diagnosis	*Total*	*Surgery*	*Irradiation*	*Both*
Non-functioning pituitary adenoma	45	42	22	22
Craniopharyngioma	23	19	11	11
Prolactinoma	11	4	3	3
Pituitary apoplexy	4	1	0	0
Rathke's cyst	3	3	0	0
Astrocytoma	4	2	4	2
Medulloblastoma	4	4	4	4
Miscellaneous	37	20	19	13

Miscellaneous: Cushing's disease, acromegaly, empty sella syndrome, arachnoid cysts, chordoma, idiopathic isolated GHD, glioblastoma, thalamic tumour, meningioma, Langerhan's cell histiocytosis, HSV encephalitis, pineal tumour, septo-optic dysplasia, nasopharyngeal neuroblastoma

Table 4.2: Other medical conditions and hormone replacement of the GHD patients (n=131)

Replacement therapy	**N (%)**
Corticosteroids	92 (68%)
Thyroxine	79 (58%)
Sex Hormones	85 (63%)
DDAVP	28 (21%)
Dopamine Agonists	17 (13%)
Total hormone replacement	20 (15%)
Coexisting Medical Conditions	
Diabetes Mellitus	5 (4%)
Hypertension	36 (27%)
Epilepsy	12 (9%)
Dyslipidaemia	74 (56%)
Previous CVA	6 (5%)
Ischaemic Heart Disease	7 (5%)
Asthma	6 (5%)
Obesity	5 (4%)
Osteoarthritis	4 (3%)
Anxiety/depression	3 (2%)
Severe COPD	2 (2%)
Peripheral Vascular Disease	1 (1%)

Table 4.3: Comparison of the measured parameters in the patients with GHD between *d3* genotypes

Measured variables	GHR genotype fl/fl	d3/fl or d3/d3	p value
n	72	59	
Gender (male: female)	37:35	34:25	
Age (years)	49 (12)	51 (12)	NS
Peak GH (mU/l)	2.7 (3.4)	2.5 (2.2)	NS
GH dose (mg)	0.5 (0.6)	0.4 (0.2)	NS
Pre-treatment IGF-1 (nmol/l)	13 (5)	11 (4)	NS
Present IGF-1 (nmol/l)	26 (12)	26 (12)	NS
QoL			
AGHDA	9 (6)	11 (7)	NS
HADS-depression	6 (4)	7 (5)	NS
HADS- anxiety	6 (4)	7 (5)	NS
LFSS	42 (4)	41 (4)	0.02
Satisfaction scale	25 (5)	25 (5)	NS
Disease impact scale	26 (7)	24 (7)	NS
VAS energy scale (cm)	7.5 (5)	6.0 (2)	NS
Measurements			
Height (SDS)	-0.51 (1.4)	-0.23 (1.2)	NS
BMI (SDS)	2.06 (1.1)	2.25 (1.0)	NS
WHR	0.92 (0.1)	0.94 (0.1)	NS
Body Fat (%)	34.2 (8.8)	35.7 (10.2)	NS

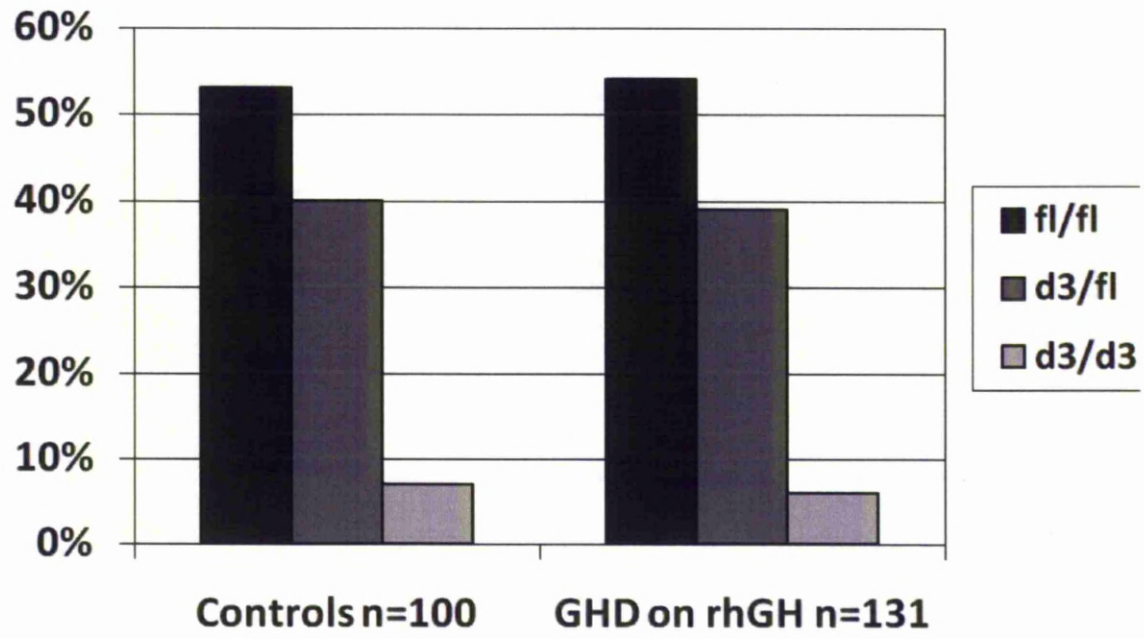
Significance $p<0.05$, NS - not significant. Data are presented as mean (SD)

Table 4.4: Comparison of the measured parameters between the GHD patients and controls

	Controls (n=100)	GHD on treatment (n=131)	p value
Gender (male:female)	44:56	71:60	
Age (years)	45 (13)	50(12)	0.008
QoL			
AGHDA	5(6)	10(7)	<0.0001
HADS-depression	3(3)	7(5)	<0.0001
HADS- anxiety	6(4)	6(4)	NS
LFSS	43(5)	42(4)	0.02
Satisfaction scale	21(4)	25(5)	<0.0001
VAS energy scale (cm)	7.1(2.1)	5.7(2.0)	<0.0001
Measurements			
Height (SDS)	-0.24(1.1)	-0.39(1.3)	NS
BMI(SDS)	1.22(1.0)	2.14(1.0)	<0.0001
WHR	0.87(0.1)	0.93(0.1)	<0.0001
Body Fat (%)	29.8(9.5)	34.9(9.5)	<0.0001

Significance $p<0.05$, NS - not significant, Data are presented as mean (SD)
HADS - hospital anxiety and depression scale, LFSS- life fulfilment and satisfaction scale, VAS- visual analogue scale, BMI- body mass index, WHR- waist-hip ratio.

Figure 4.1: The frequency of the different GH receptor polymorphisms in the patient and control populations



CHAPTER 5

POLYMORPHISM OF EXON 3 EXPRESSION IN THE CONTROL POPULATION

Introduction

Adult height distribution in normally growing populations of both sexes presents a wide range of variation (23 cm in women and 27 cm in men)(251, 252) and differences between populations with different ethnic backgrounds have also been described(253, 254). The question arises as to whether the *d3/fl*-GHR polymorphism would contribute as a factor, among others, to explain these differences. Several studies have characterised the relationship between the growth hormone receptor exon 3 genotype and the growth response in children with short stature or growth hormone deficiency undergoing rhGH therapy(3, 223, 224) but only a handful have investigated the relationship between the genotype and final adult height in a normal population(255-258). The frequency of this polymorphism has been reported in these control cohorts but none have been able to conclusively demonstrate an association between the height in an adult control population with heights normally distributed (ACPNH) between −2 SD score (SDS) and +2 SDS and the exon 3 genotype. There is however evidence to suggest geographical and ethnic variations with studies aiming to show the effects of GHR exon 3 genotypes on the height differences among populations revealing that the frequency of d3 allele was high among the Caucasian population (215, 242, 249), but low in Asian populations (259, 260). Millar et al (258) compared an African Beninese population, which has a low mean height, and an English population in terms of GHR exon 3 genotype distribution. They observed that the Benineses had a significantly higher d3/d3 frequency (47% vs. 28%). Furthermore, d3/fl and fl/fl genotypes were also found to be higher among the Beninese than among the English population (70% vs. 47%). In this study, we evaluate 100 subjects of both sexes with heights normally distributed within normal range, between −2 and +2 SDS.

Materials and methods

Control Population

A group of 100 healthy volunteers were recruited into the study from the staff of the University Hospital Aintree. All the volunteers were white Caucasian adults. They were all required to read the Volunteer information Sheet and sign the consent form before involvement in the study. The volunteers had no significant past medical history and were not on medication that could alter GH secretion. Their body composition was measured and the GHR genotypes were determined from a sample of their peripheral blood.

Analysis of the blood sample was carried out as previously described in chapter 2.

QOL questionnaires

QoL was assessed in the control populations using the same 4 validated questionnaires as the GHD population. The Adult Growth Hormone Deficiency Assessment (AGHDA) questionnaire(159), 10-cm Visual Analogue Scale (VAS), the Hospital Anxiety and Depression (HADS) questionnaire, the Life fulfilment Scale and the Satisfaction and Disease Impact Scale adapted for GHD patients(158). The volunteers were not required to fill in the Disease Impact scale questionnaire. The order of completion of questionnaires was consistent with the GHD patients and all questionnaires were answered in a single session.

Genotyping and Hormone assessment

Genomic DNA was isolated from peripheral blood lymphocytes by standard procedures. A 35-cycle amplification of the region around exon 3 of the hGHR gene was performed on samples using specific primers (GenBank accession no. AF155912) (215) that amplify a

fragment of 935-bp for fl allele and 532-bp for d3 allele as described in chapter 2. Quality control assessment included using positive and negative controls in each batch of samples.

Statistical analysis

Results are expressed as percentages and mean ± SD. Continuous data analysis for a difference among the *GHRd3*/GHR*fl* genotypes was calculated using ANOVA (with post-hoc Bonferroni correction for multiple testing). Categorical data analysis including the *GHRd3*/GHR*fl* allelic and genotype frequencies was performed by the chi-square test. Statistical analyses were performed using SPSS15.0 for Windows.

Results

Population studies and genotype frequencies and Height (SDS) and BMI (SDS) across the control population

Of the 100 healthy adult controls, 44% were males with a mean age of 45 (±13) years. The frequency of the genotypes was 53% *(fl/fl)*, 40% *(d3/fl)* and 7% *(d3/d3)* (table 5.1)

The mean QoL-AGHDA score was 5(±6) and the mean VAS score was 7.1(±2.1) cm. The anxiety and depression scale scores were normal at 6(4) and 3(3) respectively (normal 0-7). Measurements of BMI and height were expressed in mean (SD) and standard deviation scores (SDS) for normal adult Caucasian population. The mean BMI was 26.45(4.3)kg/m^2 and mean body fat was 29.8(9.5)% (see table 4.4). Further analysis to determine the influence of the frequency of the genotypes on these parameters showed no significant differences between groups (table 5.2) and on the extremes of height (i.e. SDS<2.0 and >2.0); no significant difference in the extremes of height was identified.

Discussion

In this study, the prevalence of the GHR-d3 polymorphisms in the control population of 47.9% for *fl/fl*, 43.6% for *d3/fl* and 8.5% for *d3/d3* was comparable to previous studies showing about half of the population being homozygous for the *fl* allele. In our study, this polymorphism did not influence the standard deviation score for the adult height and BMI in the control population. This has been demonstrated in previous studies which have also not shown correlation between heights of the individuals and GHR isoforms (249, 256). Kenth et al (226) reported a GHR exon 3 genotype distribution similar to our study, and they also failed to demonstrate an association between the adult height and GHR genotype. Raz et al (227) investigated GHR exon 3 genotype distribution in 211 Swiss adults with normal stature. As in our study, there was no significant difference in final adult height and height-SDS according to the exon 3 genotypes.

The adult height population evaluated by us included a fairly even distribution of subjects of both sexes with heights normally distributed within normal range, between −2 and +2 SDS and as with similar studies, no statistically significant differences in mean height-SDS values among the three GHRd3 genotypes could be found. Whilst our study did not measure the serum IGF-1 of the control population to determine its relationship to the height SDS and GHR isoforms, recent evidence shows that there were no differences in genotypes, IGF-1 and IGF binding protein-3 (IGFBP-3) SDS values among control subjects grouped according to their height SDS(256).

Adult height is not determined by a single gene but by complex polygenic inheritance. Studies in adult twins have shown that differences in height are related by 75-89% to genetic factors (261, 262). Genetic influences on birth length and postnatal growth are still being studied(241). Genome-wide analysis is expected to facilitate the discovery of new genes related to somatic growth and to reveal the extent of impact of known genes. GHR

exon 3 genotypes constitute the first genetic factor shown to affect the response to GH therapy(3).

Another potential effect of this polymorphism of the GHR has been studied in non-GHD populations. A recent study in a Chinese obese population without GHD reports that the exon 3 GHR polymorphism has a significant effect on BMI with the d3 allele demonstrating a protective effect on the development of metabolic syndrome by increasing insulin sensitivity (240). Similar results were also reported in 2008 by Binder et al(242) where *d3/d3* allele was associated with weight regulation towards a lower BMI in patients with Turner's syndrome.

Further studies are required to determine the significance, if any, of this polymorphism of the GHR in healthy populations.

Table 5.1: Frequency of genotypes in the control population

Genotype	Controls n=100
fl/fl	53%
d3/fl	40%
d3/d3	7%

Table 5.2: Comparison of anthropometric measurements and BC between genotype groups in the control population

	GHR genotype		
Measured variables	**fl/fl**	**d3/fl or d3/d3**	**p value**
Frequency	53%	47%	
Height (SDS)	-0.22(1.1)	-0.23(1.1)	ns
BMI(SDS)	1.25(1.0)	1.23(1.02)	ns
Body Fat (%)	29.9(9.5)	29.8(9.4)	ns

Significance $p<0.05$, NS - not significant, Data are presented as mean (SD)

CHAPTER 6

FINAL DISCUSSION AND STUDY SUMMARY

GENERAL DISCUSSION

For GHD adults in the United Kingdom, apart from biochemical evidence of severe GHD, the decision to commence and continue treatment are based on the demonstration of impaired QoL from NICE approved validated questionnaires. Two main sets of GHD patients are seen in our adult clinic population; a treated group on rhGH and an asymptomatic group not requiring GH replacement therapy.

Despite several recent publications in the paediatric population, there have been few publications studying the role of polymorphisms in the GHR gene and response to GH therapy in the adult population. Similar studies investigating the exon 3 polymorphism in the adult population (4, 246) did not address QoL, the main indication for the use of rhGH. Exon 3 polymorphism in these GHD adults was not predictive of biochemical and hormonal response to rhGH therapy so the controversy still exists as to the role of the GHRd3 polymorphism on the response of GHD patients to treatment. Some studies in children have demonstrated a superior growth velocity in individuals with the *d3* isoform (3, 224, 241) while other studies report there is no significant difference between these groups (222, 225). We hypothesized that adult GHD patients with exon 3 deletion are less likely to be symptomatic from GHD than those without.

This project aimed to determine the prevalence of the polymorphism of exon 3 of the GHR in the local population and also to analyze the impact of the genotypes GHR-*d3/d3*, GHR-*d3/fl*, and GHR-*fl/fl* on response to treatment with rhGH in GHD adults. We therefore studied the relationship between QoL, the qualification for rhGH therapy using NICE criteria and deletion of exon 3 in the GHR gene. We also investigated the relationship between exon 3 deletion and other markers of GHD: serum IGF-1 prior to rhGH therapy and anthropometric measures.

A few studies in adults with GHD using a variety of QoL instruments have shown multiple impairments in QoL in untreated GHD adults compared to control adults. Many studies have also shown improvement in QoL measures especially energy levels and psychological well-being in the first 2-3 years following rhGH replacement (126, 171, 184). However, a few studies have failed to demonstrate this improvement (222, 225). This could be because the QoL was measured using non-specific psychometric instruments (200, 209). In studies in which questionnaires designed specifically for GHD have been used to assess QoL, improvements with rhGH therapy have been consistently reported (158, 263, 264) such as disease specific health related QoL questionnaire previously designed in this unit for use in GHD patients (158).

An interesting group of patients in whom data is lacking are the GHD patients without impairment in QoL scores. These asymptomatic GHD patients do not seem to require treatment with rhGH according to present treatment guidelines as their QoL scores are comparable to those of health controls (see chapter 3).

The prevalence of the three GHR-d3 genotypes in the population we studied was comparable to previous studies in which up to half of the population is homozygous for the *fl* allele(221, 242, 243). As previous studies in children suggest that patients who delete exon 3 on one or both alleles *(d3/fl and d3/d3)* have a similar growth response to rhGH, data from these patients were analysed together and separately. The results of our study do not support our original hypothesis that patients with the d3/fl or d3/d3 genotype are less likely to need rhGH therapy to improve quality of life than those with the fl/fl genotype.

The difference in depression scale between the two genotype groups is probably of no clinical significance because scores of 0 to 7 on the HADS-depression scale are classed as normal with 8 to 10 being borderline normal and 11 to 21 being abnormal(237). Data from

this project also suggest that the GHR-d3 polymorphism does not influence body composition neither stimulated GH levels during provocative testing prior to rhGH therapy. A total of 100 healthy Caucasian adults were studied and the prevalence of the GHR-d3 polymorphisms was comparable to previous studies showing up to half of the population being homozygous for the *fl* allele. Similar frequencies were also seen in the GHD patient population. Previous studies have shown that growth response in GHD children is greater in children who exclude exon 3 on one or both alleles compared to those who have the full length isoform on both alleles(224). Therefore, for this study, analysis was done with the genotypes GHR-*d3/fl* and *d3/d3* grouped together and also separately. We acknowledge that the low prevalence of the homozygous d3 *(d3/d3)* precludes proper study of this group. This thesis suggests that in the adult population with GHD factors other than the GHR-d3 polymorphism influence the QoL. There are multiple causes of hypopituitarism in GHD adults and patients receive multiple and prolonged treatments, attending regular and repeated follow-up MRI/CT scanning which, in themselves, generate anxiety. As a result, determinants of QoL in adults are probably multi-factorial and may in fact be difficult to relate to a single cause such as the GHR-d3 polymorphism. Coexisting medical conditions are also likely to impact on QoL. It is also important to observe that while in children, response to treatment is easily measured by growth velocity, QoL measures remain the best way of monitoring response to treatment in adults with GHD. However in many patients, QoL is difficult to measure and interpret, and may lack sensitivity.

The data show that despite adequate hormone replacement, patients on rhGH continue to have suboptimal QoL. This has been reported in previous studies showing that the QoL scores improve and then plateau after a few years on treatment (126, 167). This is not surprising as many of these GHD patients have had surgery and radiotherapy for intracranial pathologies and also have multiple hormone deficiencies and are receiving

other hormone replacements. Also, GHD patients have a significantly higher BMI and fat mass than the control population, probably in part due to the metabolic consequences of hypothalamic-pituitary disorders (247, 248). Interestingly however, these features also do not always revert to normal with hormonal replacement. As expected, there was no significant difference in the heights of the volunteers or the patients because GHD was mainly of adult onset.

To date, results relating the GHRd3 polymorphism and growth hormone response are inconclusive. Newer studies have demonstrated that GHR*d3* polymorphism has a significant effect on BMI and the metabolic parameters in obese children (240). This is a growing are of interest and further studies are needed to investigate this and other polymorphisms in both children and adults with or without GHD. In conclusion, we have demonstrated that the deletion of exon 3 in the GHR gene does not influence QoL and energy levels in adults with GHD.

These data suggest that the polymorphism in exon 3 of the GHR does not influence adult height, QoL or body composition in the normal adult control population nor the extremes of height in the control population in agreement with a previous study(249). It does not influence peak GH levels during dynamic pituitary stimulation testing prior to commencement of treatment. The polymorphism also does not influence patient response to treatment as determined by their QoL scores and body composition. Similarly, in GHD adults treated with rhGH for >1year, rhGH dose, QoL and body composition, as well as lbiochemical parameters, were not influenced by GH isoform expressed by the patient.

Recent studies have investigated the effect of the polymorphism of exon 3 of the GHR on metabolic parameters such as systolic blood pressure and BMI in obese (240), Turner's (242) and acromegalic populations (265) with conflicting results.

Other polymorphisms such as 504A>G at codon 168 of exon 6 and polymorphism 1576 A>C at codon 526 are common but do not influence response to rhGH treatment in children (225). However, the C.1319G>T polymorphism was found to be associated with therapeutic efficacy in GHD children (250). Additionally, other genes within the GH pathway may also be important, and this relative importance may differ between adults and children. Therefore, further studies to investigate genes within and outwith the GH pathway are needed. This is important as rhGH is expensive; some patients develop adverse effects from therapy, while others show little response. Individualisation of therapy in this group of patients therefore is likely to have multiple benefits.

The stimulus to embark on long-term GH replacement is likely to come from the patient rather than the clinician, and this decision might ultimately be made on the basis of any benefits experienced by the patient during a trial period of GH replacement.

There is an argument however that because GH replacement in adults has physical and biochemical effects that may be considered by a clinician to be beneficial, a patient may not necessarily perceive these benefits and none of these effects currently constitute an absolute indication for GH replacement (266). Until a time when these effects might be shown to be beneficial to the patient in the long term (for example, a reduction in mortality from cardiovascular disease or a sustained increase in bone mineral density associated with a decrease in incidence of bone fracture), the indication for GH replacement in adults is likely to be the improvement in well-being that many patients experience whilst on treatment(163).

CHAPTER 7

PUBLICATIONS AND ABSTRACTS FROM THE RESEARCH STUDY

PEER REVIEWED PUBLICATIONS

1. **Adetunji OR,** Blair JC, Javadpour M, Alfiveric A, Pirmohamed M, MacFarlane IA. Deletion of exon 3 in the growth hormone receptor gene in adults with growth hormone deficiency: comparison of symptomatic and asymptomatic patients. Clin Endocrinol (Oxf). 2010 Mar; 72(3):422-3.

2. **Adetunji OR**, MacFarlane IA, Javadpour M, Alfiveric A, Pirmohamed M, Blair JC - The d3/fl-growth hormone (GH) receptor gene polymorphism does not influence quality of life and body composition in GH- deficient adults receiving GH replacement therapy. Eur J Endocrinol. 2009 Oct; 161 (4):541-6.

PUBLISHED ABSTRACTS

1. **Poster presentation** at the Society for Endocrinology BES 2009 *16th-19th March 2009*

   Title: Expression of exon 3 of the growth hormone receptor gene in adults with growth hormone deficiency on growth hormone replacement therapy.

   Authors: **Adetunji OR**, Blair JC, Javadpour M, Alfiveric A, Pirmohamed M, MacFarlane IA

2. **Poster presentation** at the International Congress for Endocrinology, Rio de Janeiro. *8th-12th November 2008*

   Title: Does expression of exon 3 of the growth hormone receptor gene influence the quality of life of patients with growth hormone deficiency?

   Authors: **Adetunji OR**, Blair JC, Javadpour M, Alfiveric A, Pirmohamed M, MacFarlane IA

3. **Poster presentation** at the International Congress for Endocrinology, Rio de Janeiro. *8th-12th November 2008*

   Title: Exon 3 polymorphism in the growth hormone receptor gene is not related to quality of life, body composition or IGF-1 levels in growth hormone deficient adults receiving growth hormone replacement therapy.

   Authors: **Adetunji OR**, MacFarlane IA, Javadpour M, Alfiveric A, Pirmohamed M, Blair JC

4. **Poster presentation** at the Society for Endocrinology Clinical Cases Meeting, 21st February 2007.

   Title: 33year old male with Untreated Hypopituitarism due to a Suprasellar Arachnoid Cyst

   Authors: **O.R.Adetunji**, J. Blair, M. Javadpour, I.A Macfarlane

APPENDICES

APPENDIX 1

QoL-AGHDA
Quality of Life Assessment of GH Deficiency in Adults
QOL-AGHDA English 2000-02-17 OR 7064-01

LISTED BELOW ARE SOME STATEMENTS that people may make about themselves.
Read the list carefully and put a tick a box marked YES if it applies to you.
Tick the box marked NO if it does not apply to you

Please answer every item. If you are not sure whether to answer YES or NO, tick whichever answer you think is most true in general.

	YES	NO
I have to struggle to finish jobs		
I feel a strong need to sleep during the day		
I often feel lonely even when I am with people		
I have to read things several times before they sink in		
It is difficult for me to make friends		
It takes a lot of effort for me to do simple tasks		
I have difficulty controlling my emotions		
I often lose track of what I want to say		
I lack confidence		
I have to push myself to do things		
I often feel very tense		
I feel as if I let people down		
I find it hard to mix with people		
I feel worn out even when I've not done anything		
There are times when I feel very low		
I avoid responsibilities if possible		
I avoid mixing with people I don't know		
I feel as if I'm a burden to people		
I often forget what people have said to me		
I find it difficult to plan ahead		
I am easily irritated by other people		
I often feel too tired to do things that need doing		
I often have to force myself to stay awake		
My memory lets me down		

APPENDIX 2

The Life Fulfilment Scale Adapted For Growth Hormone Deficiency

Listed below are various aspects of life. People disagree about how important each aspect is. We would like to know how important you feel each aspect to be, regardless of whether or not it applies to you personally.

For each item, please circle the number which indicates your feelings about the importance of that item. For example, if you feel that a good family is very important, circle 4: if you think it is fairly important, circle 3; and so on.

Please answer all the items.

	Very important	Fairly important	Not very important	Not at all important
a) A good family life	4	3	2	1
b) Having close friends you can confide in	4	3	2	1
c) Being able to do things you enjoy in your spare time	4	3	2	1
d) Enjoying a good social life	4	3	2	1
e) Being in good health	4	3	2	1
f) Being happy with yourself as a person	4	3	2	1
g) Being happy with the area where you live	4	3	2	1
h) Having housing which meets your needs	4	3	2	1
i) Having an adequate standard of living	4	3	2	1
j) Having enough money to do most of the things you want to do	4	3	2	1
k) Being happy with your appearance	4	3	2	1
l) A fulfilling sexual relationship	4	3	2	1

Satisfaction Scale

Now we would like to know how satisfied you are with your own life.
For each question below, please circle the number which shows best how you feel.
Please answer every question.

1) How satisfied are you, in general, with your life?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

2) How happy do you feel about the number of close friends you have – that is friends you feel you can confide in?
   a) Very happy.....1
   b) Fairly happy.....2
   c) Not very happy.....3
   d) Not at all happy.....4

3) How much do you feel able to do things you enjoy in your spare time?
   a) Often 1
   b) Sometimes.....2
   c) Rarely.....3
   d) Never.....4

4) How satisfied are you, in general, with your social life?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

5) How would you describe your health now?
   a) Excellent.....1
   b) Good.....2
   c) Fair.....3
   d) Poor.....4

6) How happy are you with the way you feel about yourself?
   a) Very happy.....1
   b) Fairly happy.....2
   c) Not very happy.....3
   d) Not at all happy.....4

7) How satisfied are you in general with the area that you live?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

8) How satisfied are you in general with your present housing condition?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

9) How satisfied are you with your present standard of living?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

10) How satisfied are you with the amount of money you have coming in?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

11) How satisfied are you with your sexual relationship?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

12) How satisfied are you with your appearance?
   a) Very satisfied.....1
   b) Satisfied.....2
   c) Dissatisfied.....3
   d) Very dissatisfied.....4

Impact Scale

We would like to know how much you feel your condition and its treatment affects your everyday life.
For each item listed, please circle the number which shows best how you feel.

1) Your relationship with your spouse/partner?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4
   e) Does not apply (no spouse/partner).....5

2) Your relationship with other close members of your family?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4

3) Your social life and activities?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4

4) Whether or not you are able to work in paid employment?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4
   e) Does not apply (not working).....5

5) The kind of paid work you can do?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4
   e) Does not apply (not working).....5

6) Your health overall?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4

7) Your relationship with friends?
   a) A lot 1
   b) Some.....2
   c) A little.....3

d) Not at all.....4

8) The way you feel about yourself?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4

9) Your plans and ambitions for the future?
   a) A lot.....1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4

10) Standard of living?
   a) A lot 1
   b) Some.....2
   c) A little.....3
   d) Not at all.....4

APPENDIX 3

Visual Analogue Scale for Energy Levels

How much energy do you feel you have today?

Place a vertical line on the on the scale below to indicate how much energy you have today.

No energy _____ Full of energy

APPENDIX 4

The Hospital Anxiety and Depression Scale (HADS)

You are being asked to choose one response from the four given. You are required to give an immediate response and not dwell too long about their answers.
The questions relating to anxiety are marked 'A' and to depression marked 'D'
The score for each answer is given in the right column.
Please circle the most appropriate answer.

Please answer all questions

A	**I feel tense or 'wound up':**	
	Most of the time	3
	A lot of the time	2
	From time to time, occasionally	1
	Not at all	0
D	**I still enjoy the things I used to enjoy:**	
	Definitely as much	0
	Not quite so much	1
	Only a little	2
	Hardly at all	3
A	**I get a sort of frightened feeling as if something awful is about to happen:**	
	Very definitely and quite badly	3
	Yes, but not too badly	2
	A little, but it doesn't worry me	1
	Not at all	0

D **I can laugh and see the funny side of things:**

As much as I always could	0
Not quite so much now	1
Definitely not so much now	2
Not at all	3

A **Worrying thoughts go through my mind:**

A great deal of the time	3
A lot of the time	2
From time to time, but not too often	1
Only occasionally	0

D **I feel cheerful:**

Not at all	3
Not often	2
Sometimes	1
Most of the time	0

A **I can sit at ease and feel relaxed:**

Definitely	0
Usually	1
Not Often	2
Not at all	3

D **I feel as if I am slowed down:**

Nearly all the time	3
Very often	2
Sometimes	1
Not at all	0

A	**I get a sort of frightened feeling like 'butterflies' in the stomach:**	
	Not at all	0
	Occasionally	1
	Quite Often	2
	Very Often	3
D	**I have lost interest in my appearance:**	
	Definitely	3
	I don't take as much care as I should	2
	I may not take quite as much care	1
	I take just as much care as ever	0
A	**I feel restless as I have to be on the move:**	
	Very much indeed	3
	Quite a lot	2
	Not very much	1
	Not at all	0
D	**I look forward with enjoyment to things:**	
	As much as I ever did	0
	Rather less than I used to	1
	Definitely less than I used to	2
	Hardly at all	3
A	**I get sudden feelings of panic:**	
	Very often indeed	3
	Quite often	2
	Not very often	1
	Not at all	0

D **I can enjoy a good book or radio or TV program:**

Often	0
Sometimes	1
Not often	2
Very seldom	3

Scoring

(Add the As = Anxiety. Add the Ds = Depression). The norms below will give you an idea of the level of Anxiety and Depression.

0-7 = Normal

8-10 = Borderline abnormal

11-21 = Abnormal

APPENDIX 5

Patient Information Leaflet

1 Introduction

We would like to invite you to take part in a research study being conducted at the University Hospital Aintree. This is absolutely voluntary and if after reading this information leaflet, you decide not to participate, your decision will be accepted without question. Your treatment will not be affected in any way if you decide not to take part in the study.

This information sheet describes the study and what it would mean for you if you decide to participate.

2 What is the purpose of the study?

There is some evidence to suggest that the response to GH therapy in patients who are deficient of this hormone may be determined by our genes (the basic building blocks of life). The type of gene you express may determine how severe your symptoms are and how well you respond to the replacement treatment and how much GH you will require to get reasonable response. It is likely that knowing which genotype you belong to will help you and others like you to quickly determine the dose and of GH required for therapy instead of long months of titrating therapy. The purpose of this study is to identify polymorphism of this gene and to compare it with changes in your body composition and QoL. We require your permission to be able to test for this gene.

3 Why have I been chosen?

You have been chosen for the study because you are deficient in a hormone called growth

hormone. Growth hormone stimulates growth in children but for fully grown adults who are deficient in this hormone, it alters body composition, their general well-being and energy levels. We need to recruit a large number of patients such as yourself. It is likely that about 400 will be required. In view of this, we consider this to be a long-term study lasting up to 2years.

4 Who is organising the study?

This is a joint study between the endocrine clinic at the University Hospital Aintree and the University of Liverpool.

What will happen to me if I participate?

If you decide to participate in this study, an extra 10mls (equivalent to two teaspoons-full) will be taken when you have your routine blood taken for tests at the clinic. Taking a blood tests is a routine procedure with minimal risks such as minor discomfort at the time of taking blood and bruising. You will be required to answer standardised questionnaires, one of which is routinely administered for all patients with laboratory evidence of growth hormone deficiency according to NICE guidelines. We will also require some data relating to previous blood test results from your notes (the information we need is detailed in the attached proforma).

When starting GH replacement therapy for the first time, regular routine blood tests are done to determine your response to GH therapy until normal levels of GH in the blood is reached. Participants will also have repeat questionnaires administered to determine their QoL after normalization of their blood test. Both the blood tests and questionnaires are normal routine procedure for all patients on GH therapy. For the purpose of this study however, additional questionnaires will be administered to the routine questionnaire filled

before and after optimizing treatment with GH.

What are the possible benefits of taking part?

Determination of the polymorphism of the GHR gene as it relates to response to GH therapy may not benefit you directly. It may however help future GH deficiency patients to determine who is likely to respond to therapy and the dose of GH most appropriate for which genotype of GHR gene. GHR gene polymorphism maybe then be routinely checked before commencing therapy. This will prevent delay in reaching normal GH levels in the blood, prevent repeated blood tests and make funding more easily available for the patients who are most likely to benefit from therapy.

What are the possible risks of taking part?

The extra 10ml of blood taken from your routine tests will not cause any significant problems as your body normally contains about 5 litres of blood (5000ml). All your results will be kept completely confidential and it will not be possible to link your results to you, so there will be NO effect on you (or your family's) ability to obtain any kind of insurance.

What will happen to my blood test?

Your blood sample will be stored in a locked freezer in the University Hospital Aintree and/or the University of Liverpool. It is important to note that the stored blood samples and any notes relating to it will be identified only by a code number, and therefore it will not be possible to trace the blood sample back to you by persons not involved with the study. We will keep your sample until all of it is used up.

Your blood sample will be considered to be a gift to the University Hospital Aintree, which will act as custodian of all the samples obtained as part of this project. In some cases, a

small amount of your sample will be provided to other researchers either in the UK or other parts of the world. However, it is important to remember that this will only be identified by a code number.

In the short-term, it is unlikely that the samples will be of any commercial value to the Trust. However, it is possible that there may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to findings in a group of patients rather than from samples from a single patient.

Confidentiality – who will know I am taking part in the study?

As stated above, your sample will be anonymised, and therefore the genetic information obtained from it will be kept strictly confidential and will only be available to the study doctors. Other nominated members of the study can only access this on written request to you. If you would require your GP be involved about your participation in the study, we willdo so in writing. We will disclose individual results to yourself and your GP only with your permission.

We will combine all the results from the many patients taking part in the study, and publish any important results in medical journals.

Contact for further information

If you need further information or are worried about any aspect of the study, please do not hesitate to contact Dr 'Lara Adetunji, Research Fellow University Hospital Aintree on 0151 529 6146 or Dr I.A. MacFarlane, Consultant Physician University Hospital Aintree on 0151 529 4650.

RESEARCH PARTICIPANT CONSENT FORM

Patient study ID Number _____

I FREELY AGREE TO PARTICIPATE IN THIS STUDY	Yes	No
I have read the Patient Information Sheet provided	Yes	No
I have had the opportunity to ask questions about the study	Yes	No
I understand that my medical care will not be affected if I do not participate in the research study.	Yes	No
I give consent for my case records to be looked at if necessary for purposes of the study.	Yes	No
I can withdraw at any time without giving a reason	Yes	No
I give permission for my GP to be informed	Yes	No

Name and signature of Participant:

.... **Date:**.....

Name and signature of Principal/Local Investigator:

.... **Date:**.....

5 **GP/CONSULTANT INFORMATION LETTER**

Dear Doctor

Your patient ..... who has growth hormone deficiency has consented to participate in a study to identify the polymorphism of the growth hormone receptor gene. This study will involve adult patients from age of 16yrs onwards attending the neuroendocrine clinic under Dr I.A. MacFarlane. Blood tests will be taken to determine polymorphism of genomic DNA that encodes for growth hormone receptor gene. Quality of life questionnaires will also be administered to your patient before and after optimising therapy with GH. Your patient can withdraw at any time without giving an explanation. Your patient has been given an information leaflet and informed consent has been taken.
If you require any further information, please contact the local investigator, Dr Omolara Adetunji Tel: 0151 529 6146 or the Chief Investigator, Dr Ian MacFarlane, Tel: 0151-529-4650.

Yours faithfully

Dr 'Lara Adetunji

APPENDIX 6

Volunteer Information sheet

6 Introduction

We would like to invite you to take part in a research project. This is absolutely voluntary and if when you have heard about the study, you would prefer not to participate, your decision will be accepted without question. This is an information sheet, which describes the project and what it would mean for you if you did participate.

7 What is the purpose of the study?

There is evidence to suggest that about one half of the human population have differences in the gene (building blocks of the body) that determines the function of the growth hormone receptor. Whilst this difference does not affect the growth and development of the population, it affects how patients with growth hormone deficiency respond to treatment. The purpose of this study is to identify how common these different genes are in the normal local population (such as yourself) and compare this to the frequency in a population of patients with deficiency of growth hormone.

8 Why have I been chosen?

You have been chosen at random for this study because your age and sex match those of the patients involved in the study.

9 Who is organising the study?

The study is being organised by the University Hospital Aintree NHS trust and the University of Liverpool.

What will happen to me if I take part?

If you decide to participate you will be asked to provide between 10ml of blood (equivalent to two teaspoons-full). You will be asked to answer questionnaires pertaining to your energy levels along with some basic measurements to check your weight, height, hip and waist measurements and your body's percentage fat mass. Taking blood tests is a routine procedure with minimal risks such as minor discomfort at the time of taking blood and bruising.

What will happen to my blood test?

Your blood sample will be stored in a locked freezer in the University Hospital Aintree. We will isolate DNA from your blood sample, which will then be used to analyze genes involved in the control of the growth hormone receptor. It is important to note that the DNA isolated from the blood sample and any notes relating to it will be identified only by a code number initially. Once this has been done, your sample will be irretrievably anonymised at which time it will not be possible to trace the blood sample back to you. We will keep your sample until all of it is used up.

Your blood sample will be considered to be a gift to the University Hospital Aintree, which will act as custodian of all the samples obtained as part of this project. In some cases, a small amount of your sample will be provided to other researchers either in the UK or other parts of the world. However, it is important to remember that only a code number will identify this.

In the short-term, it is unlikely that the samples will be of any commercial value to the Trust. However, it is possible that there may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to findings in a group of patients rather than from samples from a single patient.

What are the possible benefits of taking part?

You will not receive any financial or therapeutic benefit from taking part in the study. However, the knowledge gained from this study may go a long way to help patients with growth hormone deficiency in the future.

What are the possible risks of taking part?

There may be some minor but short-lasting discomfort from having a blood test. Since all your results will be kept completely confidential, and it will not be possible to link your results to you, there will be NO effect on your (or your family's) ability to obtain any kind of insurance.

Confidentiality – who will know I am taking part in the study?

As stated above, your sample will be anonymised, and therefore the genetic information obtained from it will be kept strictly confidential and not be disclosed to anyone. We will not disclose individual results to yourself, your GP or your hospital doctor. We will combine all the results from the many participants taking part in the study, and publish any important results in medical journals.

Contact for further information

If you need further information or are worried about any aspect of the study, please do not hesitate to contact Dr 'Lara Adetunji, Research Fellow University Hospital Aintree on 0151 529 6146 or Dr I.A. MacFarlane, Consultant Physician University Hospital Aintree on 0151 529 4650.

THANK YOU FOR READING THIS LEAFLET.

RESEARCH PARTICIPANT CONSENT FORM

Volunteer study ID Number _____

I FREELY AGREE TO PARTICIPATE IN THIS STUDY	Yes☐	No☐
I have read the Volunteer Information Sheet provided	Yes☐	No☐
I have had the opportunity to ask questions about the study	Yes☐	No☐

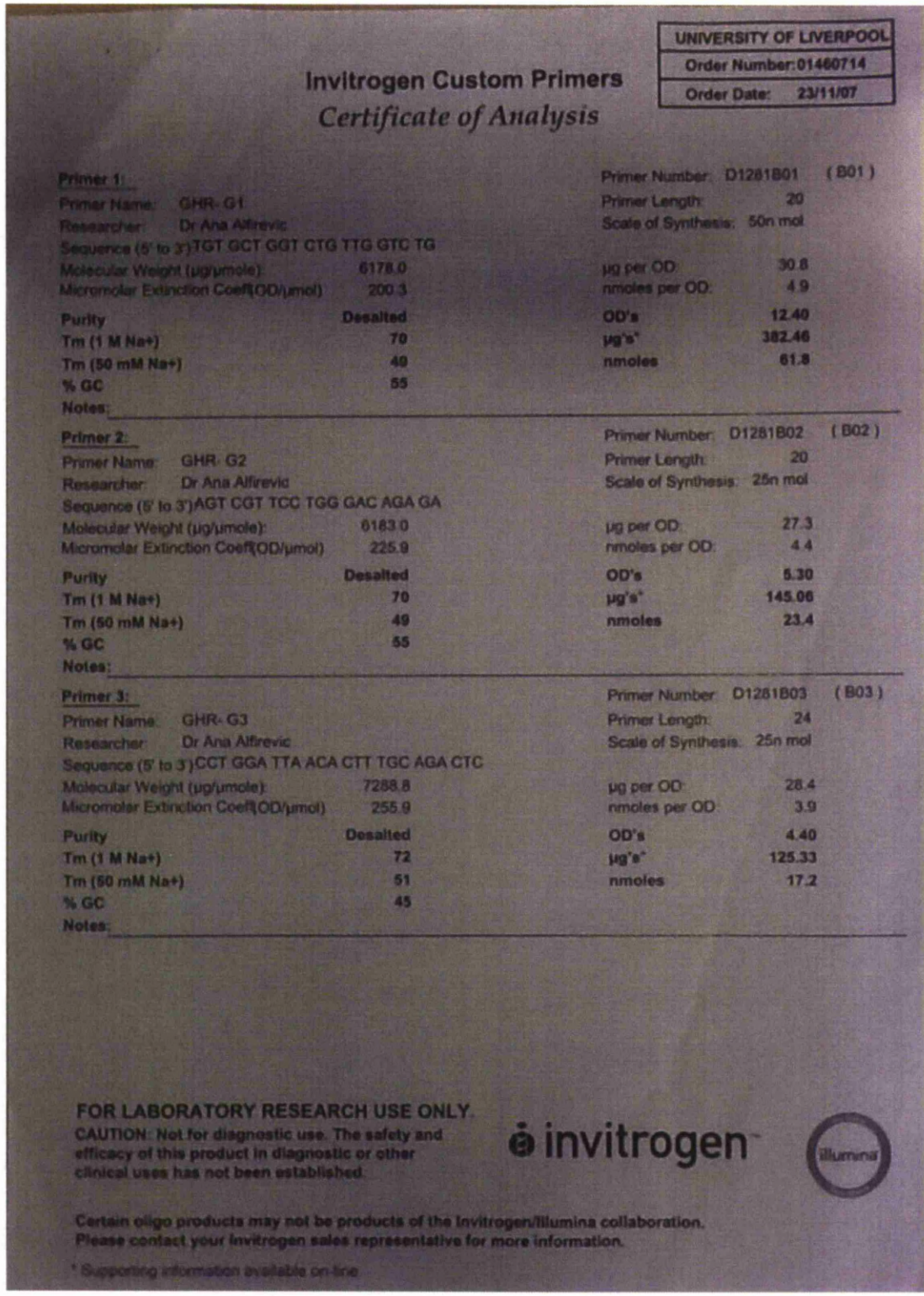
Signature of Participant:

…… ….. **Date:**……

Name and signature of Principal/Local Investigator:

…… ….. **Date:**……

APPENDIX 7: CERTIFICATE OF ANALYSIS OF PRIMERS



UNIVERSITY OF LIVERPOOL
Order Number:01460714
Order Date: 23/11/07

Invitrogen Custom Primers
Certificate of Analysis

Primer 1:
Primer Name: GHR- G1
Researcher: Dr Ana Alfirevic
Sequence (5' to 3'):TGT GCT GGT CTG TTG GTC TG
Molecular Weight (µg/µmole): 6178.0
Micromolar Extinction Coeff(OD/µmol) 200.3
Purity: Desalted
Tm (1 M Na+): 70
Tm (50 mM Na+): 49
% GC: 55
Notes:

Primer Number: D1281B01 (B01)
Primer Length: 20
Scale of Synthesis: 50n mol
µg per OD: 30.8
nmoles per OD: 4.9
OD's: 12.40
µg's*: 382.46
nmoles: 61.8

Primer 2:
Primer Name: GHR- G2
Researcher: Dr Ana Alfirevic
Sequence (5' to 3'):AGT CGT TCC TGG GAC AGA GA
Molecular Weight (µg/µmole): 6183.0
Micromolar Extinction Coeff(OD/µmol) 225.9
Purity: Desalted
Tm (1 M Na+): 70
Tm (50 mM Na+): 49
% GC: 55
Notes:

Primer Number: D1281B02 (B02)
Primer Length: 20
Scale of Synthesis: 25n mol
µg per OD: 27.3
nmoles per OD: 4.4
OD's: 5.30
µg's*: 145.06
nmoles: 23.4

Primer 3:
Primer Name: GHR- G3
Researcher: Dr Ana Alfirevic
Sequence (5' to 3'):CCT GGA TTA ACA CTT TGC AGA CTC
Molecular Weight (µg/µmole): 7288.8
Micromolar Extinction Coeff(OD/µmol) 255.9
Purity: Desalted
Tm (1 M Na+): 72
Tm (50 mM Na+): 51
% GC: 45
Notes:

Primer Number: D1281B03 (B03)
Primer Length: 24
Scale of Synthesis: 25n mol
µg per OD: 28.4
nmoles per OD: 3.9
OD's: 4.40
µg's*: 125.33
nmoles: 17.2

FOR LABORATORY RESEARCH USE ONLY.
CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

invitrogen

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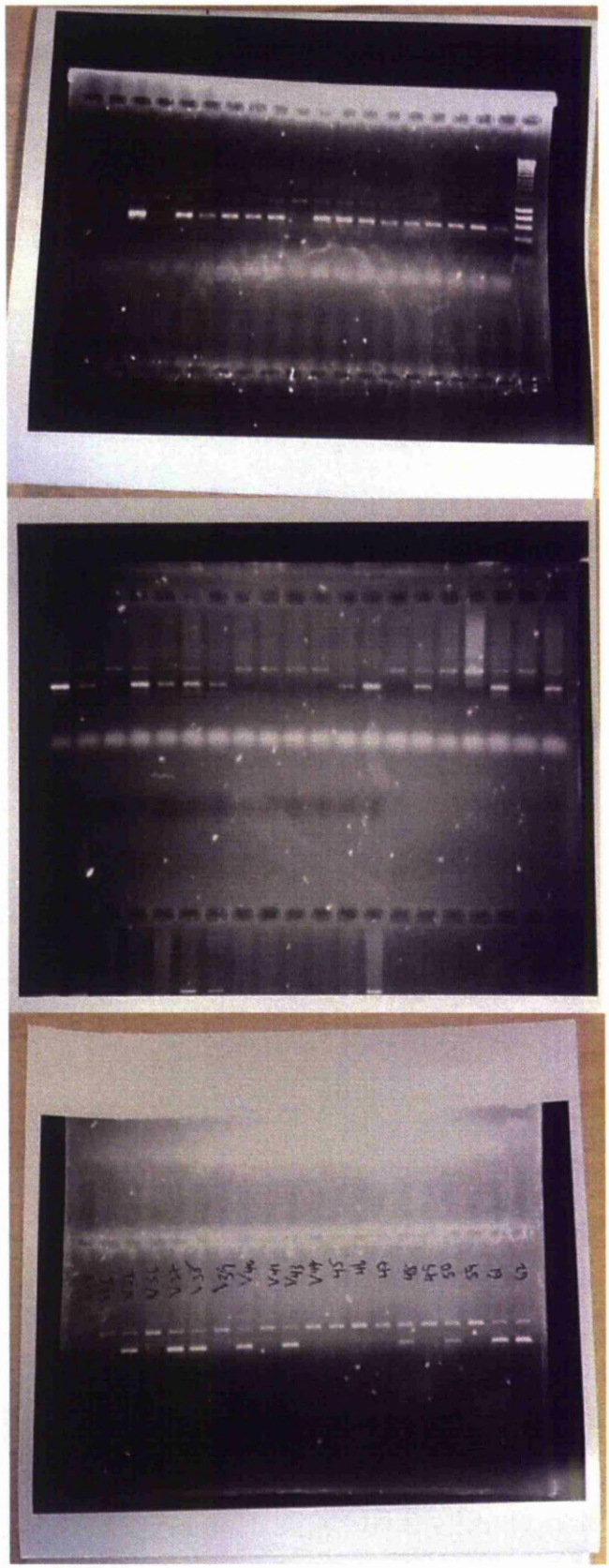
Certain oligo products may not be products of the Invitrogen/Illumina collaboration. Please contact your Invitrogen sales representative for more information.

* Supporting information available on-line

APPENDIX 8: Some pictures of amplified GHRd3 alleles following electrophoresis



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